JP-4 AND JP-9 FUEL TOXICITY STUDIES USING FRESH WATER FISH AND AUFWUCHS

ANNUAL REPORT

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AEROSPACE MEDICAL RESEARCH LABORATORY
AEROSPACE MEDICAL DIVISION
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AMRL-TR-76-50

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FOR THE COMMANDER

Anthony A. Thomas

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Director
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20. ABSTRACT *(Continue on reverse side if necessary and identify by block number)*

This report contains the results of research efforts concerned with defining the effects of potential environmental contamination resulting from the use of certain Air Force materials on fresh water fish and aufwuchs. Materials evaluated include RJ-4, RJ-5, Methylcyclohexane, JP-9 and JP-4. Techniques for exposing organisms to those substances are discussed and the results of such exposures are presented.

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SUMMARY AND CONCLUSIONS

1. This report deals with the toxicity to fish and attached periphyton growths ("aufwuchs") of the fuels JP4 and JP9 and the JP9 components RJ4 (isomers of tetrahydro-methylcyclopentadiene dimer), RJ5 (isomers of tetrahydro-norbornadiene dimer; Shelldyne H), and MCH (methylcyclohexane).

2. Gas chromatographic methods preceded by solvent extraction techniques have been developed for the quantitative analysis of JP9 and its components in aqueous systems and in fish tissues. Excellent recoveries of all materials from water and fish flesh were obtained.

3. The response of the Golden Shiner (Notemigonus chrysoleneas) was examined in static bioassays of fuel in water emulsions, of water in contact with pools of floating or sunken fuel, and of water saturated with but not directly in contact with fuel.

a. The toxicity of static pools of unemulsified fuel and fuel components was orders of magnitude less than the emulsified materials as assessed by the 96-hr LC 50 values:

Fuel	96-hr LC 50, µℓ/ℓ		
	Fuel in Water Emulsion	Water in Contact with Fuel	Water Soluble Components of Fuel
JP4	18	570	200,000
JP9	2	470	--
MCH	70	240	23,500
RJ4	0.5	100	--
RJ5	0.6	4,700	--

The vast decrease in toxicity from JP4 emulsion to water in contact with JP4 to water soluble components of JP4 is indicative of the importance of the physical presence of the fuel and of the nature of its physical state in water to determining toxic effects on fish. The JP4 data would indicate that the majority of the toxicity could be removed from an aquatic system by removing droplets or pools of pure JP4.

b. Evidence existed to suggest that fish deaths in assays of water in contact with fuel layers or droplets was in some degree due to physical contact between fuel and fish.

c. Apparently because of the importance of physical contact in determining toxic response, it was not possible to accurately predict the toxicity of JP9 from that of its components when 24-hr renewal of emulsions were used to combat volatility losses in a 96-hr static assay. Accurate prediction of JP9 toxicity from its components' toxicity is possible when no emulsion renewal is conducted.

d. The toxicity of emulsions did not depend on water hardness in the range of hardness from 38 to 123 mg $CaCO_3/\ell$.

4. The use of a Mount Brungs dilution device for dosing continuous-flow bioassay tanks with aqueous extracts of fuel results in considerable volatility losses and in carryover of fuel droplets into bioassay exposure tanks. These factors combined with variable water solubility can strongly influence the composition of a multi-component fuel (such as JP9) so that the composition of the material to which fish are exposed is greatly different from the composition of the pure fuel.

5. In continuous-flow bioassays using the flagfish (Jordanella floridae) aqueous solution concentrations of 0.83 mg/ℓ MCH and 0.2 mg/ℓ RJ4 had no effect on egg hatchability; aqueous solution RJ5 concentrations of greater than 0.05 mg/ℓ and JP9 levels in excess of 0.23 mg/ℓ reduced flagfish egg hatchability. It is tentatively concluded that the effect of JP9 solutions on flagfish egg hatchability could be largely ascribed to its RJ5 content. MCH aqueous concentrations of 0.83 mg/ℓ did not affect flagfish fry development as assessed by fish length or weight. A short-term experiment (7 days) allowed the level of MCH causing a lethal response to flagfish to be set between 0.83 mg/ℓ and 1.85 mg/ℓ.

6. In continuous-flow bioassays using the rainbow trout (Salmo gairdneri), non-lethal aqueous concentrations of RJ4 were found to be below 0.03 mg/ℓ; the 97-day LC 50 value was 0.045 mg/ℓ. Non-lethal aqueous concentrations of RJ5 were below 0.04 mg/ℓ; the 97-day LC 50 value was 0.072 mg/ℓ. The non-lethal aqueous concentration of MCH appeared to be below 0.8 mg/ℓ; an approximate 23-day LC 50 value was 1.3 mg/ℓ. The non-lethal level of JP9 (of composition similar to that obtained in the bioassay exposure tanks, i.e., 85.4% MCH, 1.8% RJ4, 13.3% RJ5) was some value less than 0.37 mg/ℓ. The 23-day LC 50 value was 0.38 mg/ℓ.

7. It was possible to accurately predict the toxicity of aqueous JP9 solutions from the toxicity of aqueous solutions of its components RJ4, RJ5 and MCH by the toxic unit method of Sprague. In making this prediction it was important to use the composition of JP9 in the aqueous solution in the exposure tank rather than the composition of the JP9 used to prepare the aqueous fuel solution because of the changes in composition caused by different relative solubility and volatility.

8. In experiments on the accumulation of fuels in fish tissues, it was found that the flagfish can tolerate an MCH total body burden of some 0.5 mg MCH/g wet weight without lethality. A lethal response to MCH exists somewhere in the range of total MCH body burdens of 0.5-1.19 mg MCH/g wet weight. The rainbow trout and the flagfish appear to concentrate MCH from their aqueous surroundings to about the same extent (150-fold); rainbow trout concentrate RJ4, 9800 times, and RJ5, 3900 times from aqueous solution. Body burdens of 0.43 mg RJ4/g and of 0.27 mg RJ5/g wet fish will produce 50% mortality of rainbow trout. Surviving rainbow trout exposed to the JP9 components MCH, RJ4 and RJ5 will rapidly void MCH from their tissues but RJ4 and RJ5 are retained.

9. Aufwuchs techniques need more refinement before highly satisfactory results can be obtained in the fresh water environment. In a series of studies with JP4, a correlation of 60% was obtained relating the suppression of Aufwuchs photosynthetic index (PI) to concentration of JP4. At 1000 µℓ/ℓ concentration of emulsified fuel, suppression of PI was 43% for JP4, 35% by RJ4 and 25% by RJ5. Water extracts of fuel were far less suppressive in that undiluted extracts produced the following PI reductions: MCH—90%, JP4—59%, RJ5—22%, and RJ4—3%.

PREFACE

The research reported herein was conducted at the Sanitary Engineering Research Laboratory, University of California at Berkeley. Professors R. C. Cooper and David Jenkins were the Co-Principal Investigators. Mr. Stephen Klein was the project engineer. Ms. P. C. Ulrichs was responsible for conduct of bioassays. Masters candidates Mr. Jonathan Palm, Ms. Charlene Kawamura and Ms. Nancy Quan aided in the development and conduct of gas chromatographic analysis and bioassay procedures.

TABLE OF CONTENTS

TABLE OF CONTENTS (continued)

LIST OF TABLES

LIST OF FIGURES

I. INTRODUCTION

The purpose of this study is to provide information for the assessment of environmental effects of materials such as fuels and fuel components as used by the United States Air Force.

This report presents the results of fish toxicity studies on the jet fuels JP4 and JP9 and the components of JP9—RJ4, RJ5 and MCH. Fish toxicity was measured in batch and continuous-flow systems using both warm water fish (Jordanella floridae and Notemigonus chrysoleneas), cold water fish (Salmo gairdneri), and attached periphyton growths (aufwuchs). One can visualize that there are several ways in which a fuel may be introduced into the aquatic environment and come in contact with aquatic biota. Thus a fuel spill may result in the formation of floating or sunken pools of pure fuel as well as emulsions of fuel droplets and fuel components in true solution. Surface runoff from areas exposed to fuel may also contain these three physical forms of fuel components. Waste discharges from fuel processing and manufacturing may also contain this variety of physical forms of fuel. Because of these possibilities, toxicity studies were conducted on static pools of fuel, fuel emulsions, and aqueous fuel solutions. In addition to measurements of fish toxicity, as assessed by deaths of adult fish, experiments were conducted on the effect of fuel components in fish bodies and its relationship to toxic response was examined. The voiding of fuel components from fish placed in fuel-free water was also studied.

As a part of these studies developmental work was conducted on the extraction, concentration and gas chromatographic analysis of fuel components in water and fish flesh. It was also necessary to devise modifications of dosing and dilution apparatus for conducting continuous bioassays on volatile and poorly water-soluble compounds.

II. MATERIALS AND METHODS

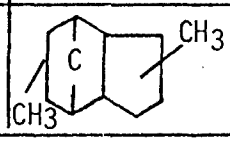
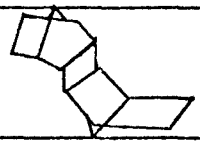
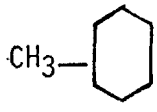
Fuels

The studies reported herein concentrate on the jet fuel JP9 which has three major components: RJ4 (a mixture of isomers of tetrahydro-methylcyclopentadiene dimer), RJ5 (a mixture of isomers of tetrahydro-norbornadiene dimer —also known as Shelldyne H), and MCH (methylcyclohexane). The JP9 and its components RJ4 and RJ5 were supplied by the Aerospace Medical Research Laboratory, USAF. MCH was technical grade supplied by J. T. Baker, Co. Some properties of the fuel components are presented in Table 1. The JP9 fuel supplied varied in composition between batches. The first batch on which static bioassays were conducted contained weight percentages of 40% MCH, 15% RJ4 and 45% RJ5. The second batch on which continuous-flow bioassays were conducted contained weight percentages of 27% MCH, 16.2% RJ4 and 56.8% RJ5.

Some studies are reported on the fuel JP4 which is a complex mixture of aliphatic and aromatic hydrocarbons. The batch supplied contained weight percentages of 0.3% benzene, 0.2% toluene and 0.8% mixed xylenes.

Table 1

PHYSICAL PROPERTIES OF
FUELS AND FUEL COMPONENTS

Property	RJ4	RJ5	MCH	JP4
Boiling Point °C	221.6	272.2	100-102	--
Vapor Pressure at 170°F, mmHg	0.354	0.025	--	--
Density, g/mℓ	0.925	1.0813	0.772	0.746
Empirical Formula	$C_{12}H_{20}$	$C_{14}H_{20}$	C_7H_{14}	--
Formula Weight	164	188	98	--
Representative Chemical Structure				--

Emulsified Fuels

Emulsified fuels and fuel components for static bioassays were prepared by blending 10 mℓ fuel with 990 mℓ tap water for 1 min in a stainless steel Waring Blender. The emulsion so produced was transferred to a 2-ℓ separatory funnel and allowed to separate for 1 hr. The middle portion was taken as the sample for static bioassay of "emulsified fuels." In these assays the fuel concentration was expressed as μℓ fuel emulsified/ℓ assay vessel contents.

Non-Emulsified Fuels

Non-emulsified fuels and fuel components were examined in static bioassays by exposing fish to dilution water in contact with a floating or bottom pool of fuel. In these assays the test fish were introduced into the assay vessels before the fuel was added so that direct contact between fish and floating fuel was avoided. In these assays fuel concentration was expressed as μℓ fuel/ℓ assay vessel contents.

Water Soluble Fuels

Two methods were used for preparing water soluble fuels. For static bioassays, water was saturated with fuel by stirring overnight with 13.6% by volume of fuel in a 5-gal capacity carboy equipped with a bottom spigot. The aqueous layer was drawn off and used to prepare the dilution for bioassay.

For continuous-flow bioassays tap water, dechlorinated by passage through a 25-gal bed of activated carbon housed in a 50-gal stainless steel drum, fed two constant head tanks which in turn supplied two fuel-contacting chambers, each consisting of 12-ℓ Florence flasks that contained 1.2 ℓ each of pure fuel or fuel components and were stirred slowly with magnetic mixers. The water, flowing at a rate of 2 ℓ/min, contacted with fuel, fed to a separation carboy, and thence to a final head tank (Figure 1). In the experiments on rainbow trout, synthetic wool was placed in the fuel separation chamber to prevent carryover of fuel droplets which was evident in the earlier studies on flagfish. From this final head tank the water "saturated" with fuel passed to a proportional diluter (Esvelt and Conners, 1971), a modification of the design of Mount and Brungs (1969), that could be set to deliver dilutions of fuel "saturated" water over the entire range from 0% to 100%. Figure 2 shows the head tanks, fuel contacting chambers, separation carboys and final head tanks of two of the units located on the upper platform and two of the proportional diluters mounted vertically.

Continuous-Flow Bioassay Exposure Tanks

Continuous-flow bioassay exposure tanks (Figure 3) were of stainless steel construction, 4 ft long x 1 ft wide x 1 ft deep and were filled with removable size 40-010 mesh stainless steel screens that enabled compartmentalization of the tanks (Figure 4). An adjustable and removable standpipe allowed maintenance of a variable water depth that was usually 8 in, giving an exposure tank capacity of 80 ℓ. Nominal hydraulic residence time in the assay tanks was 6 hr.

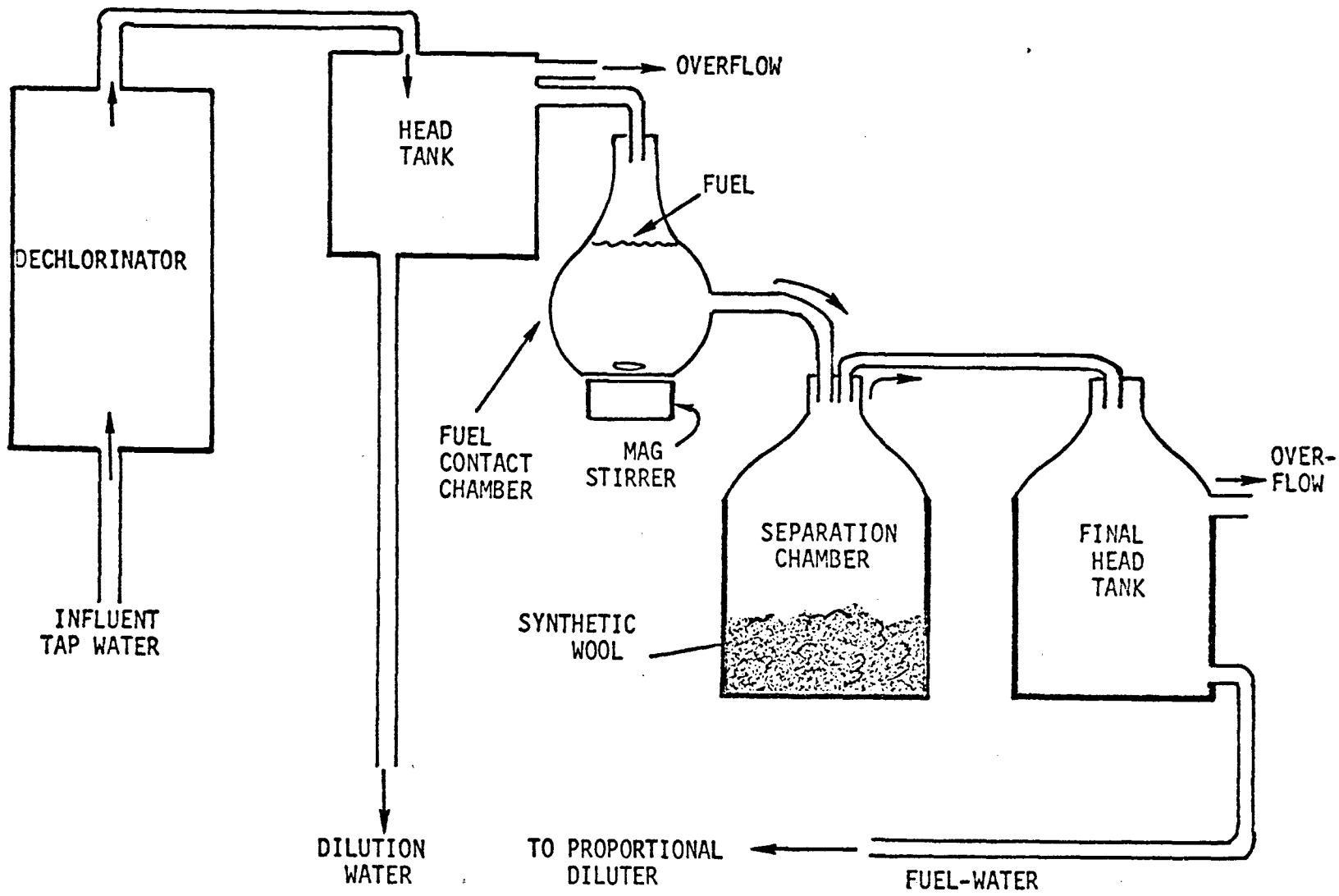


FIGURE 1. DIAGRAM OF DOSING APPARATUS FOR "SOLUBLE" FUEL COMPONENTS IN CONTINUOUS FLOW BIOASSAY

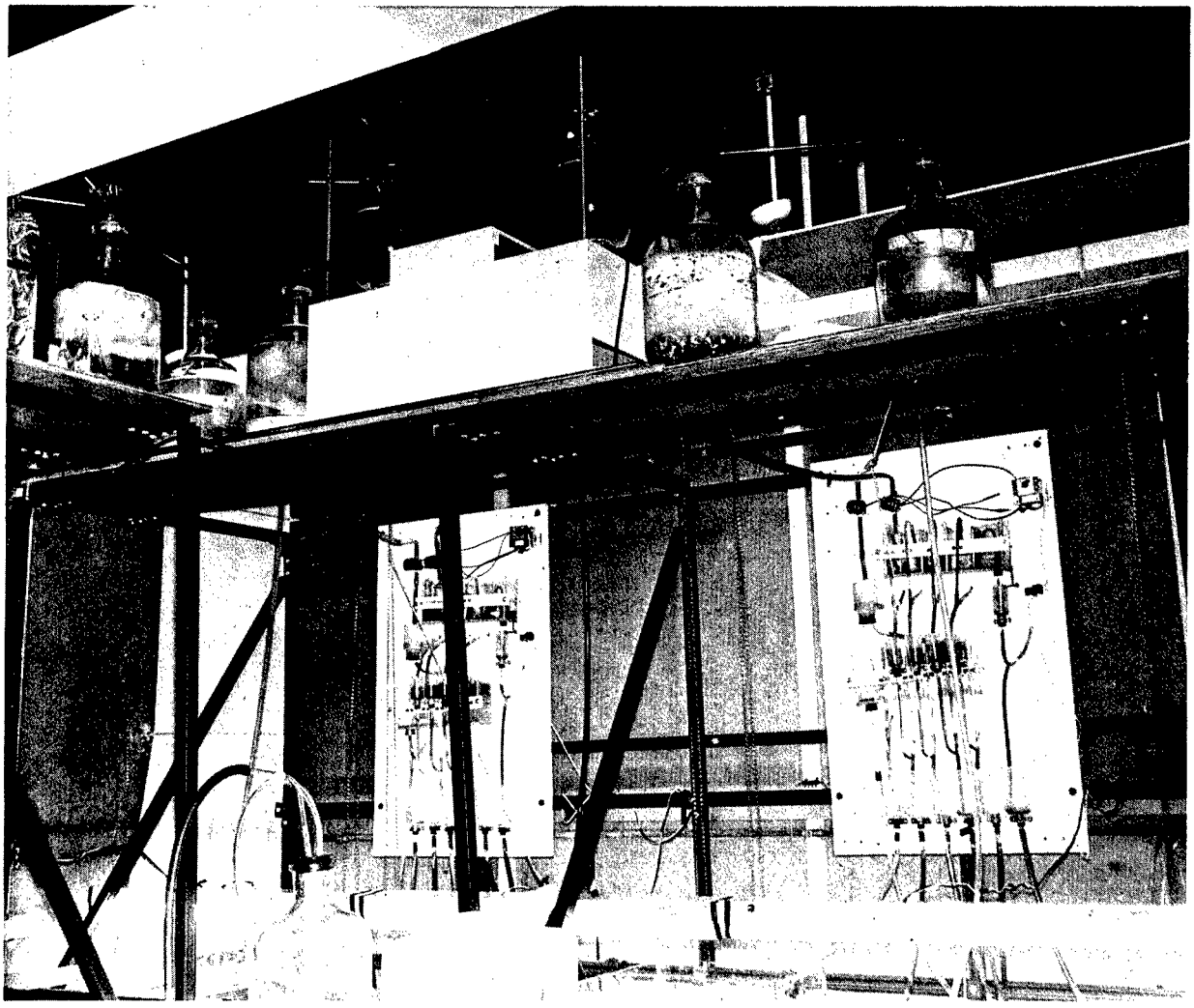


FIGURE 2. FUEL-WATER MIXING APPARATUS, SEPARATION CARBOYS (UPPER PLATFORM), PROPORTIONAL DILUTERS (VERTICALLY MOUNTED)

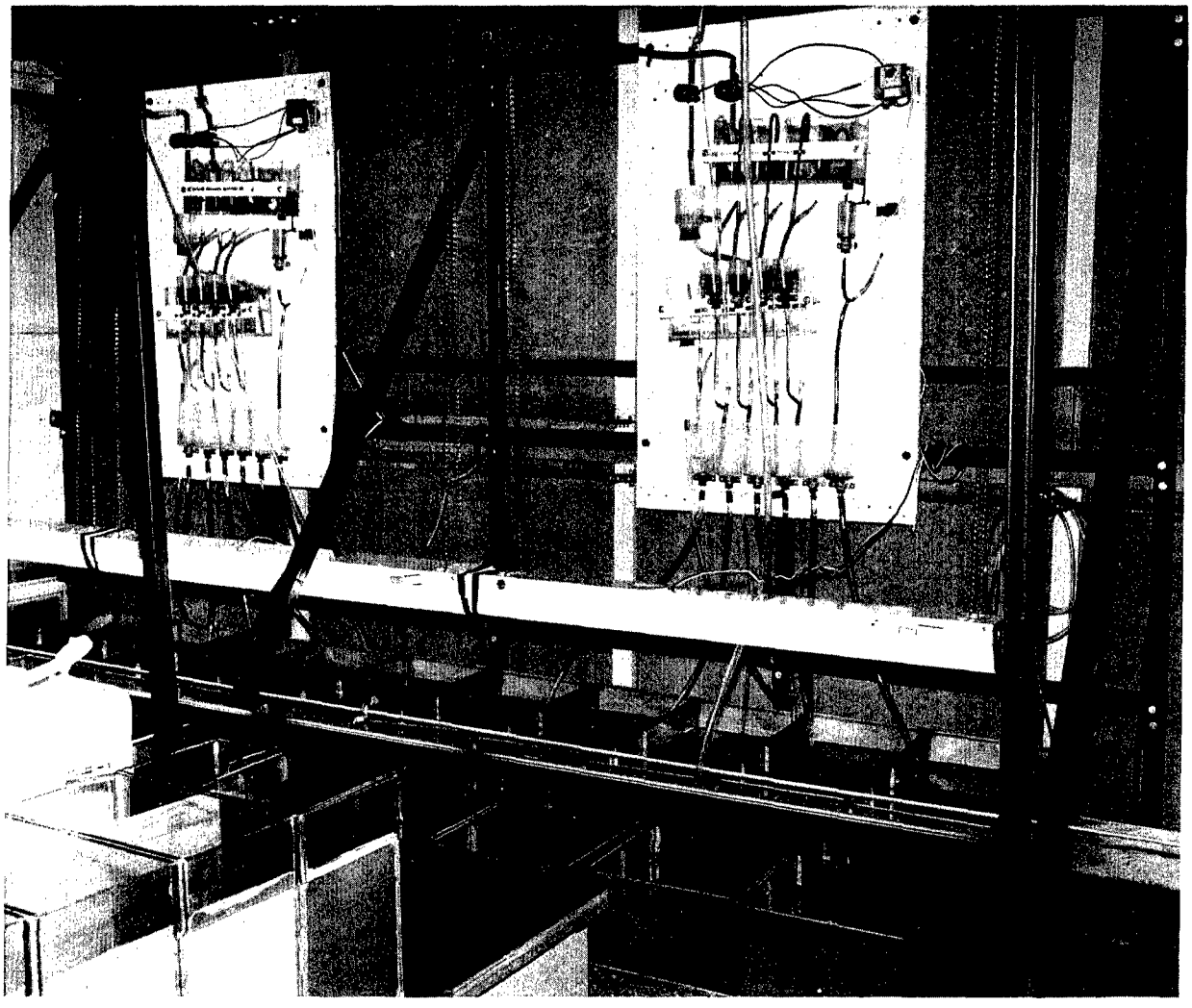


FIGURE 3. GENERAL VIEW OF PROPORTIONAL DILUTERS AND CONTINUOUS-FLOW TANKS

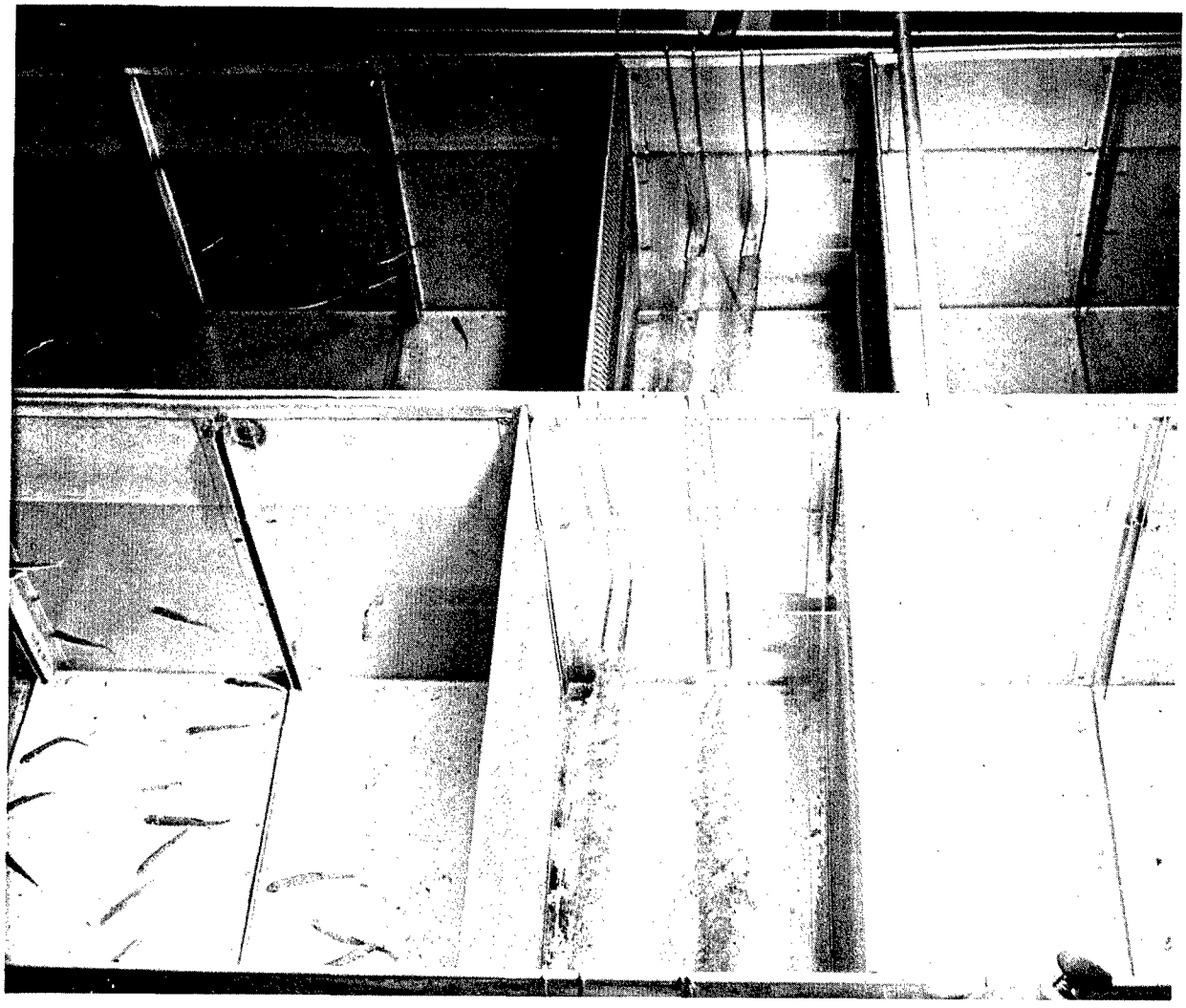


FIGURE 4. SCREENED COMPARTMENTS IN CONTINUOUS-FLOW TANKS

Egg Cups

Egg cups for egg hatchability studies were 4-oz glass jars with their bottoms replaced with a size 40-010 mesh stainless steel screen.

Fry Chambers

Fry chambers were glass chambers (12 in long, 6 in wide and 12 in deep), with size 40-010 mesh stainless steel screens at each end to allow free circulation of water. The chambers were designed to fit into the fish exposure tanks and to provide protection for and allow photographic monitoring of fry. In exposure experiments they were located about 1 ft from the inlet end of the exposure tank so that the incoming liquid provided good mixing and gentle agitation for the fry.

Static Bioassays

Static bioassays were conducted in 20ℓ-capacity wide-mouth glass jugs filled to 15 ℓ and provided with the minimum amount of aeration with filtered air to maintain the dissolved oxygen concentration above 4 mg/ℓ throughout the bioassay. The fish weight/dilution water ratios specified in Standard Methods (1970) were observed.

LC 50 Determination

LC 50 values were determined by both the Standard Methods (1970) technique and by the Reed-Muench interquartile range method (Woolf, 1968). The two techniques gave virtually identical results, but the Reed-Muench method had the advantage of allowing computation of 95% confidence limits.

Flagfish

Flagfish (Jordanella floridae) were obtained from a commercial aquarium and were selected for warm water chronic bioassays because they are easy to sex, easy to mate and have a relatively short egg-to-egg cycle. They are considered to be a species of intermediate sensitivity.

Flagfish Spawning Aquaria—were located in a room isolated from the bioassay room and maintained at 22°C, the optimal temperature for breeding. Dechlorinated water flowed through the aquaria at a rate of approximately one residence time per day and was removed by pumps. The water was continually filtered by a commercial pump-filtration system and aerated with air pumps that dispensed air through air stores. One male and five females were placed in both a 15-gal and a 26-gal capacity spawning aquarium together with a substrate consisting of dark green yarn wrapped around stainless steel cloth. Debris was removed from the aquaria daily and the aquaria were thoroughly cleaned once per week by vigorous sponging of the sidewalls and bottom. Disease was controlled by malachite green applications.

Flagfish Breeding—was encouraged by the 1:5 ratio of males to females and the presence of the substrate which served to receive fish eggs. Eggs

were collected daily from this substrate and hatched in a separate container which received a higher concentration of malachite green than aquaria containing the adults.

To promote growth and breeding, the adults were fed live brine shrimp twice per day and green tetra food once or twice per day. The fry were fed the naplii of brine shrimp on a schedule of about once per 90 minutes during working hours.

The fish were shielded from disturbances by placing paper on the side of the aquaria that faced the aisle of the room. Traffic in the room was minimized and workers were cautioned to wear dark clothing and to avoid making noise or sudden movements. The room was lighted 24 hours a day to avoid the trauma induced by turning the lights off and on.

Golden Shiners

Golden Shiners (Notemigonus chrysoleneas) were obtained from a commercial fish hatchery and were acclimated to dechlorinated Richmond Field Station tap water for one month in 300-gal capacity holding tanks prior to experimental work.

Rainbow Trout

The first-feeding fry of the rainbow trout (Salmo gairdneri) were obtained from the American River Fish Hatchery of the California State Department of Fish and Game. Before use in bioassays, the fish were acclimated for one month to dechlorinated Richmond Field Station tap water.

Expression of Fuel Concentrations

The fuel concentration for static bioassays on emulsified fuels were computed on a volumetric basis by preparing 1% by volume fuel emulsions, equivalent to a fuel concentration of 10,000 µℓ/ℓ, and making serial dilutions of the emulsions expressed as µℓ fuel/ℓ dilution water. This method of expression was also used for static bioassays on pure fuels which formed floating or sunken pools in the bioassay vessels. This method of concentration expression was admittedly not ideal but gas chromatographic analytical methods had not been developed at this stage of the work. In the continuous flow bioassays the gas chromatographic technique was available so that fuel and fuel component concentrations could be expressed on a mass concentration (mg/ℓ) basis.

Gas Chromatographic (GC) Analysis of Fuels and Fuel Components

Fuel and fuel component concentrations in water and fish flesh were determined by gas chromatographic analysis using a Fisher Model 4800 chromatograph with dual flame ionization detectors and 20 ft x 1/8 in o.d. stainless steel columns of 10% SE 30 on 80/100 Chrom W. Dual column operation permitted use of temperature programming without baseline drift from

column bleed. Accessory GC equipment included a Fisher Series 5000 Recordall recorder and an Autolab Minigrator for digital integration of peaks.

A considerable amount of development was conducted on the gas chromatographic techniques. Besides the necessary construction of standard curves relating peak area to mass of each of the components, RJ4, RJ5 and MCH, work was performed on extraction and isolation of each component from water and from fish flesh. The following procedures were developed:

Standard Curves—for each of the components, RJ4, RJ5 and MCH, are presented in Figures 5, 6 and 7, respectively, under the operating conditions specified. The results indicate a linear response in the range of 10^{-5} to 10^{-8} g.

Internal Standards—The internal standards selected for each component of JP9 are n-heptane for MCH, n-undecane for RJ4 and n-tetradecane for RJ5.

Chromatograms—Under the operating conditions given in Table 2, RJ4 was found to have four major components (Figure 8) which made up some 94% of the total peak area observed. The RJ5 had three major components (Figure 9) which always made up over 98% of the total peak area observed. MCH had only one major peak under the conditions used in this gas chromatographic work.

Table 2

NORMAL GC OPERATING CONDITIONS

Single Column Operation
Injection Port Temperature = 230°C
Detector Temperature = 280°C
H_2 Rotameter Reading = 4.5
Column temperature and carrier flow dependent on fuel as follows:

Fuel	Isothermal Temperature, °C	N_2 Rotameter
MCH	60	2.5
RJ4	150	3.0
RJ5	180	5.0

Retention Times—of the peaks for each fuel component and standard were the following:

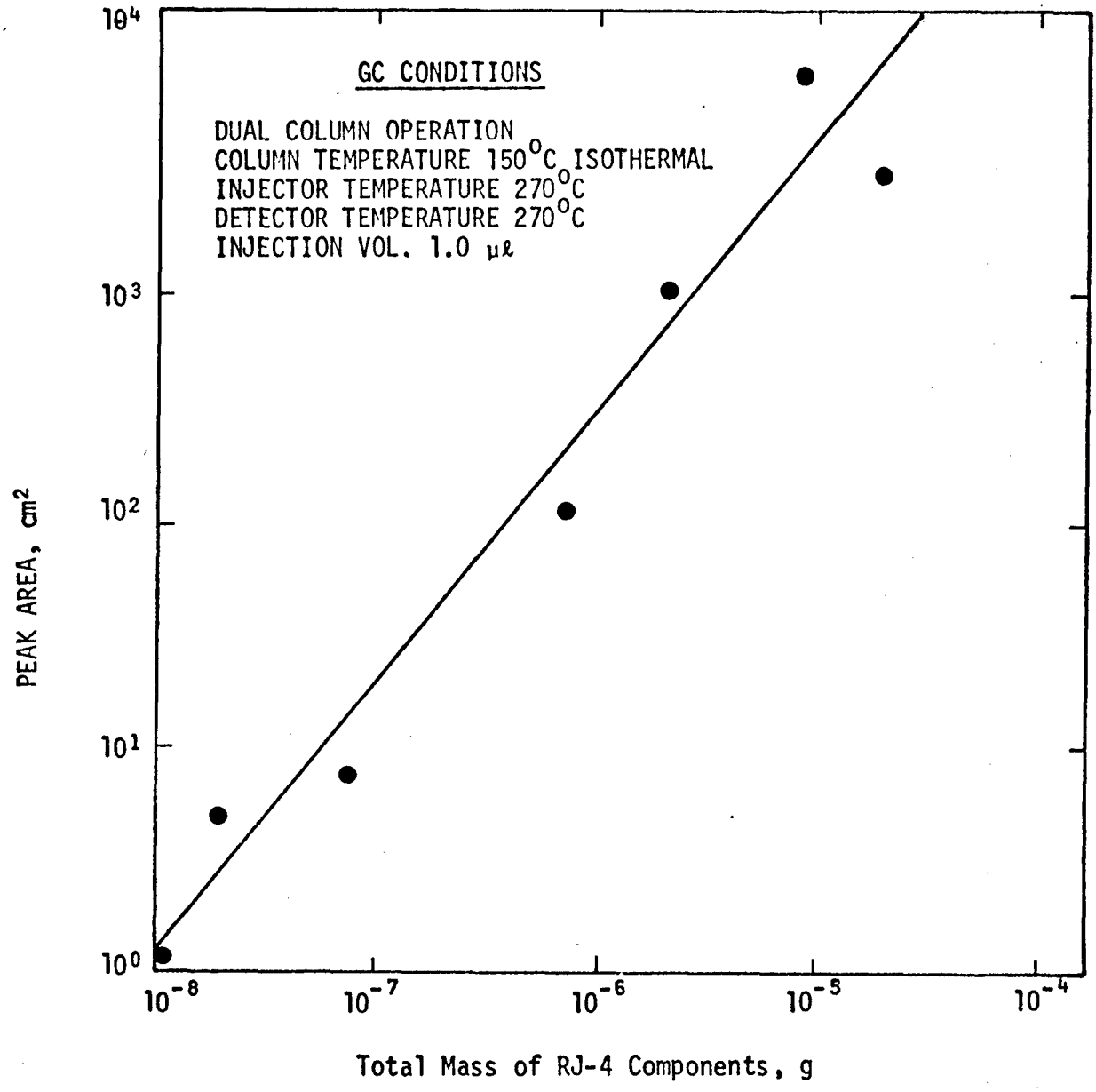


FIGURE 5. RJ-4 STANDARD CURVE

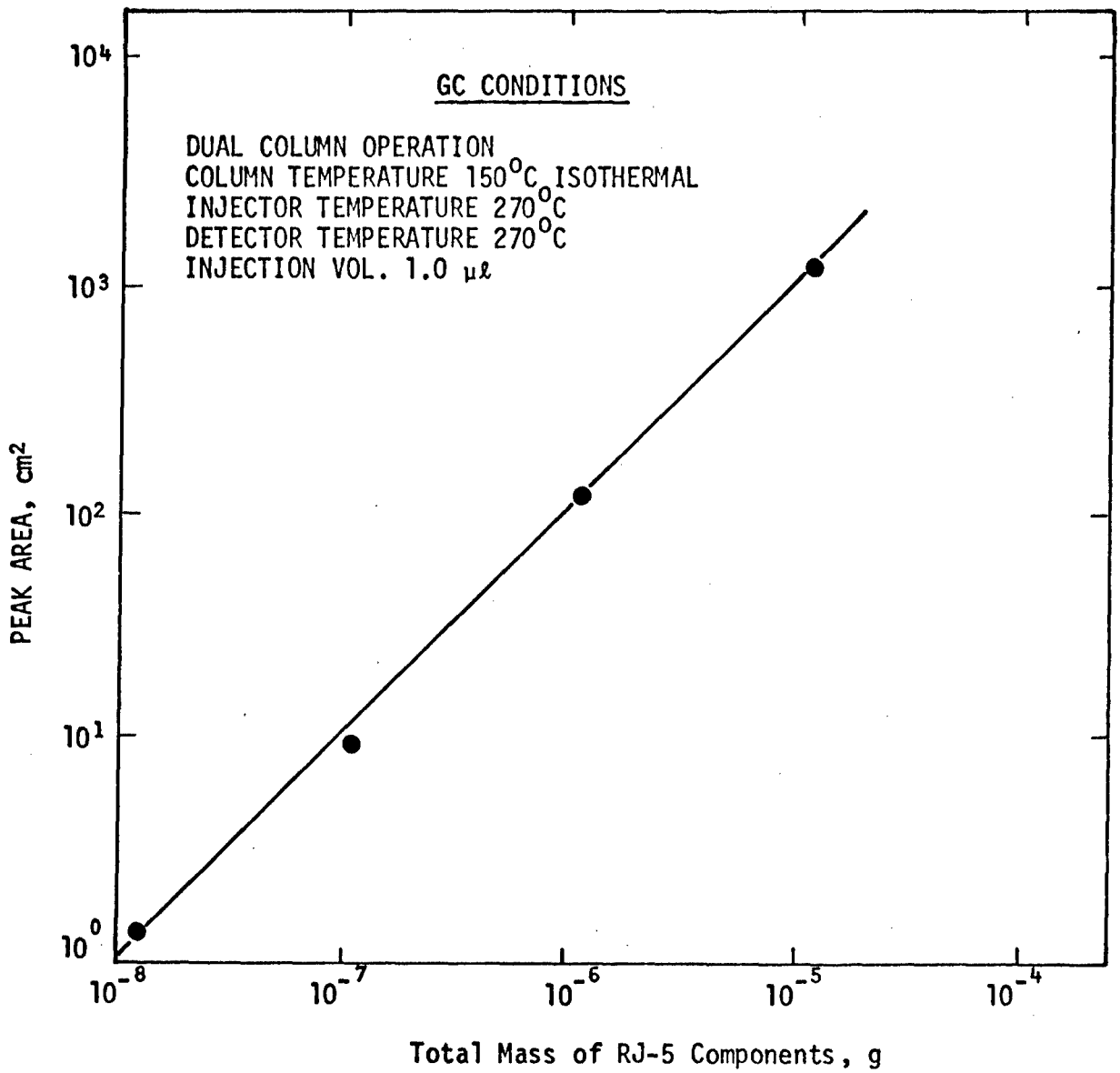


FIGURE 6. RJ-5 STANDARD CURVE

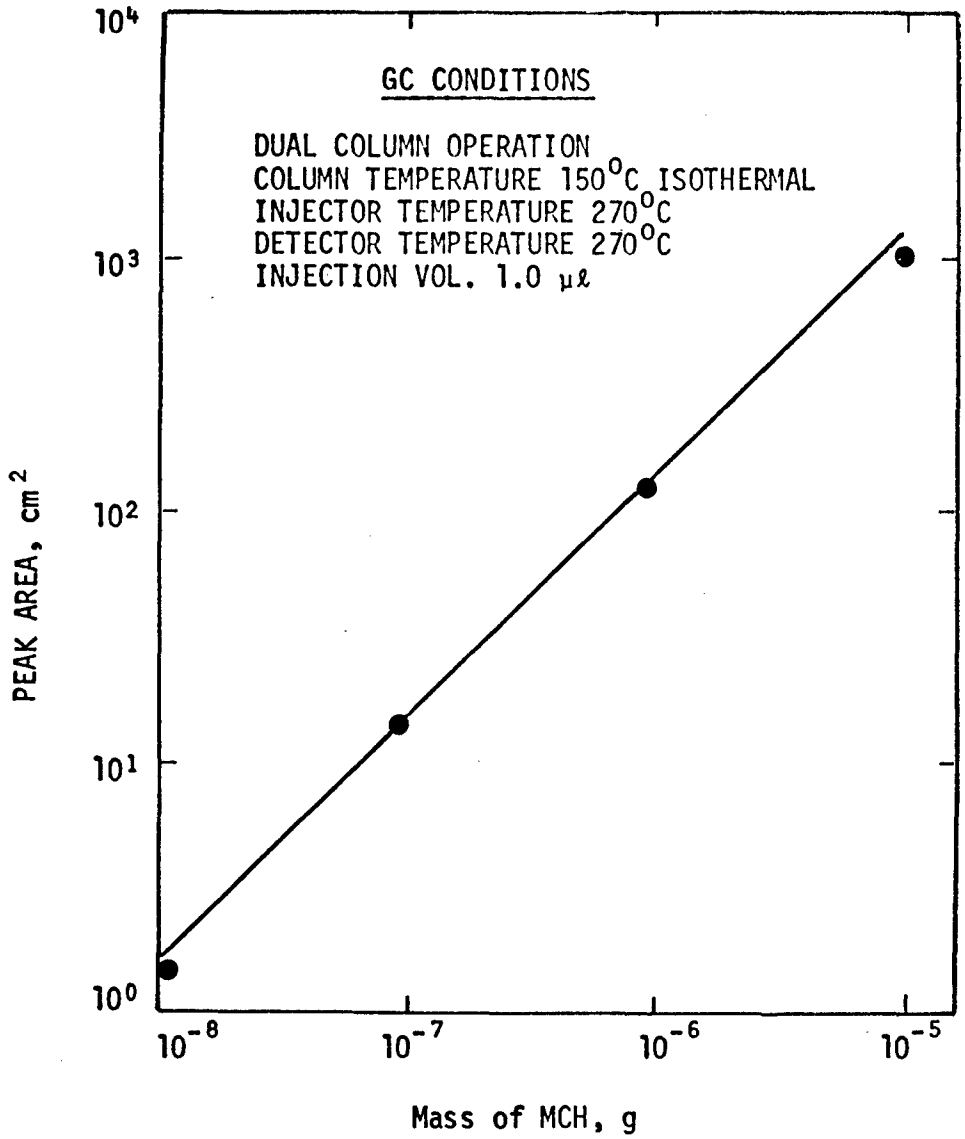


FIGURE 7. MCH STANDARD CURVE

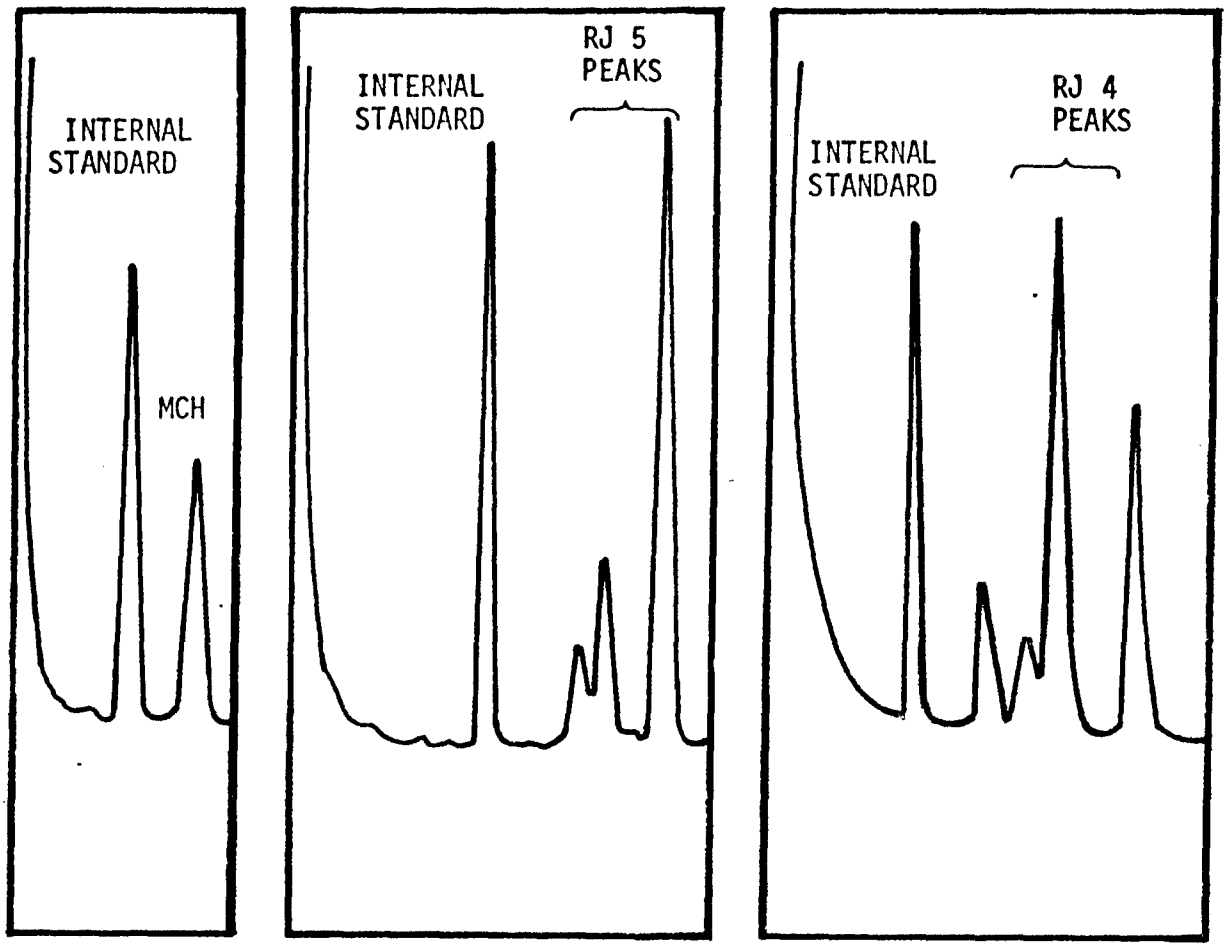


FIGURE 8. CHROMATOGRAMS OF MCH, RJ-5 AND RJ-4

Compounds	Peak	Retention Time, sec.
MCH	1	931
n-Heptane		639
RJ4	1	735
	2	847
	3	933
	4	1,161
n-Undecane		527
RJ5	1	1,250
	2	1,350
	3	1,590
n-Tetradecane		939

Recovery of Fuel Components by Rotary Evaporation

The concentration of pentane extracts of fuels prior to gas chromatographic analysis was investigated for its effect on JP9 components. Evaporation from 50 mℓ to 1 mℓ was conducted at 20°C and 0.0001 mmHg vacuum for MCH, RJ4 and RJ5 in the presence of their respective internal standards, n-heptane, n-undecane and n-tetradecane. Recoveries were as follows: MCH, 39%; n-heptane, 42%; RJ4, 95%; n-undecane, 95%; RJ5, 100%; and n-tetradecane, 101%. These data indicate that these internal standards are satisfactory for the compounds they respectively reference. It should be noted, however, that in mixtures of these components, such as the fuel JP9, a variable recovery of components will be obtained so that it is necessary to add each component's internal standard prior to concentration analysis. This procedure was followed throughout the work reported herein.

Extraction of Fuel from Water

The following method for extracting fuel from water was developed:

a. To 300 mℓ sample, add 25 mℓ nanograde n-pentane and shake 2 min by movement of the separatory funnel through a 90° arc at a rate of one complete downward and upward movement per sec.

b. If necessary to avoid emulsions, add 50 mℓ salt solution (100 g NaCl/ℓ distilled water) to original sample.

c. Allow to separate for approximately 10 min, remove aqueous layer, and dewater the pentane layer by passing it through a 3-cm diam by 3.5-cm deep column of anhydrous sodium sulfate.

d. Repeat steps a and c and combine extracts.

e. Concentrate the combined extract on a rotary evaporator to either 10 mℓ or 1 mℓ (concentration factor of 30-fold or 300-fold, respectively).

f. Perform gas chromatographic analysis using the conditions cited in Table 2.

Extraction Efficiency of Fuel from Water

The efficiency of extraction of fuel from water was evaluated to determine the number of pentane extractions required to achieve 98% recovery. A typical extraction efficiency experiment conducted on RJ5 from aqueous solution is presented here:

a. Divide 600 mℓ water-saturated solution of RJ5 into two equal portions, respectively labeled Sample A and Sample B.

b. Extract Sample A five consecutive times with 25 mℓ portions of pentane and concentrate to 1 mℓ for gas chromatographic analysis.

c. Extract Sample B once with a 25 mℓ portion of pentane and concentrate to 1 mℓ for gas chromatographic analysis.

d. Extract the water remaining from Sample B four more times with 25 mℓ portions of pentane, combine the extracts, concentrate to 1 mℓ for gas chromatographic analysis. These combined and concentrated extracts are designated as Sample C.

e. Theoretically, A = B + C

The following were obtained:

Sample	Mean GC Peak Area, mv-sec	Concentration mg/ℓ
Standard	41,264	108.1 (in pentane)
A	22,891	0.200 (in H_2O)
B	19,998	0.175 (in H_2O)
C	2,405	0.021 (in H_2O)

These results provide a check on the analytical technique since the sum of B + C is 0.196 mg/ℓ—a value that is in close agreement with the value for A of 0.200.

The extraction efficiency was computed from the following equation:

$$D = \frac{(W - W_1)}{S} \frac{V}{W_1}$$

where,

D = Distribution coefficient
W = Weight of RJ5 in initial solution, mg
W_1 = Weight of RJ5 in water after 1st extraction, mg
V = Volume of water sample, mℓ
S = Volume of pentane used for extraction, mℓ

Using the data obtained in this experiment, one obtained:

$$D = \frac{(0.06 \text{ mg} - 0.0075 \text{ mg})}{25 \text{ mℓ}} \frac{300 \text{ mℓ}}{0.0075 \text{ mg}} = 84.0$$

To determine the number of extractions required for a given total efficiency of extraction, the following equation was employed:

$$W_n = W\left[\frac{V}{DS + V}\right]^n$$

where W_n = weight of RJ5 remaining in water after n extractions, mg. Thus for 99% efficiency,

$$\frac{W_n}{W} = 0.01 = \left[\frac{300}{84(25) + 300}\right]^n$$

and

$$n = \frac{\log 0.01}{\log 0.125} = 2.21$$

Similarly for 98%, n = 1.88 and for 97%, n = 1.69.

Thus under the conditions of this experiment, two extractions were sufficient to yield recoveries of RJ5 in excess of 98%.

Fuel Accumulation in Fish-Extraction Efficiency

A method was developed for the quantitative determination of fuel accumulation in fish flesh. The following presents both a description of experimental details as well as a description of a typical experiment to determine the recovery of fuel by the extraction technique (RJ4 from Golden Shiners).

a. Inject into each of 10 fish a measured volume (approximately 1 μℓ) of an RJ4 standard solution (approximately 1000 ppm).

b. Place 25 mℓ pentane into a stainless steel blender.

c. Add 10 mℓ anhydrous Na_2SO_4 and fish.

d. Run cooling water through blender for 1 min.

e. Grind for 1.5 min.

f. Pour off pentane, wash with pentane.

g. Repeat b through f steps, grinding for 1 min.

h. Pour combined extract through a thistle tube containing anhydrous Na_2SO_4 to remove water and particulate matter. Wash the Na_2SO_4 with two 10 mℓ portions of pentane.

i. Add 1 mℓ internal standard.

j. Reduce volume to 5-10 mℓ using a rotary evaporator.

k. Clean up sample by passing through a column of 25 mℓ florosil overlayered by 10 mℓ anhydrous Na_2SO_4. Wash the column twice with 25 mℓ portions of pentane.

l. Reduce volume to 1 mℓ by rotary evaporation and analyze gas chromatographically.

The following results were obtained:

Mean concentration of RJ4 solution injected into fish = 910 mg/ℓ

Volume of RJ4 solution injected into fish = 11.2×10^{-6} ℓ

∴ Mass of RJ4 injected into fish = 10.2×10^{-3} mg

Mean concentration of RJ4 in pentane extracts = 10.7 mg/ℓ

Concentration of RJ4 expected in pentane for 100% efficient extraction = 10.2 mg/ℓ

% recovery of RJ4 by extraction = 104.9%

Aufwuchs

Aufwuchs, the attached growths of periphyton communities indigenous to a certain aqueous environment, were developed on synthetic substrates suspended in the Mokelumne River near its confluence with the Sacramento River Delta in Northern California. Each synthetic substrate consisted of roughened tygon tubing, 5 cm in length and 1.2 cm in diameter, with a surface suitable for the attachment and growth of aufwuchs communities. For each aufwuchs experiment, four racks, each holding 30 substrates, were immersed to a depth of 50 cm in the river and the growths were developed over a two-week period. Aufwuchs metabolic response was determined by measuring the effect of fuels on the photosynthetic index by the method of Krock and Mason (1971). Photosynthetic Index was defined as

$$PI = \frac{O_2 \text{ produced by photosynthesis, mg/aufwuchs}}{\text{organic mass, mg/aufwuchs}}$$

or

$$PI(chl) = \frac{O_2 \text{ produced by photosynthesis, mg/aufwuchs}}{\text{chlorophyll mass, mg/aufwuchs}} .$$

Phtotosynthetic oxygen production was measured by incubating aufwuchs in pairs of individual BOD bottles in a 20°C water bath illuminated with cool white fluorescent lights to give a light intensity of 1700 ± 75 ft candles at the bottle surface. One bottle in each pair was covered with aluminum foil to exclude light. This dark bottle measured oxygen consumption due to respiration and was incubated for 4 hr. The other bottle in each pair was exposed to the light and measured net photosynthetic oxygen production (photosynthesis - respiration). Incubation time for this "light" bottle was 2 hr.

Dissloved oxygen concentration in the bottles was measured by the Azide modification of the Winkler method (Standard Methods, 1970), both before and after incubation. The mean dry weight, organic weight, and Chlorophyll a contents of the standing crop was determined on additional growth units.

Mineral Quality

All analyses of water quality were performed in accordance with Standard Methods (1970).

III. STATIC FISH BIOASSAYS

Introduction

Golden Shiners were exposed to fuels and fuel components in a series of static bioassays. The physical mode of contact between fuel and fish varied from pure fuel added to water to form a floating or sunken pool to a highly dispersed fuel-in-water emulsion to the water-soluble fraction of fuels.

The effect of fuel volatility loss on toxicity was explored by comparing bioassays in which the test solution was renewed each 24-hr to bioassays in which the initial mixtures were retained for the entire 96-hr period of the bioassay. Two levels of water hardness were studied. Water from SERL had a mean total hardness of 123 mg $CaCO_3$/ℓ; water from Berkeley, California, had a mean total hardness of 38 mg $CaCO_3$/ℓ (Table 3). Although neither of these waters could be considered "hard," for purposes of designation in this report the higher concentration is referred to as "hard" and the lower concentration as "soft."

Table 3

MINERAL CONTENT OF WATERS USED
IN STATIC BIOASSAYS ON GOLDEN SHINERS

Parameter	Source of Water		
	Mokelumne River	Richmond Field Station	Berkeley Campus
Hardness ($CaCO_3$), mg/ℓ	110	123	38
Mg ($CaCO_3$), mg/ℓ	75	8	13
Ca ($CaCO_3$), mg/ℓ	35	115	25
Cl mg/ℓ	17	22	12
Conductivity μmhos	137	242	70
Na mg/ℓ	9	0	5
K mg/ℓ	0.5	0	1.5
TDS mg/ℓ	267	186	--
SO_4 mg/ℓ	13	20	8
Alkalinity mg/ℓ	60	100	32
pH	7.9	8.0	7.5

Results

Fuel Emulsions—The results of static bioassays of emulsified fuels using the Golden Shiner as a test organism are presented in Table 4 and Figure 9. No evidence can be found for any fuel or fuel component that would support the hypothesis that water hardness in the range 38-123 mg $CaCO_3/\ell$ had any effect on toxicity. In replicate 96-hr bioassays with no fuel renewal there were apparent large variations in the LC 50 values obtained for MCH and for the fuel JP4 which was also examined in these studies. Thus the 96-hr LC 50 values for the bioassay with no fuel renewal for MCH were 81 and 53 $\mu\ell/\ell$; for JP4 values of 49, 16 and 18 $\mu\ell/\ell$ were obtained. These differences, however, were only statistically significant for JP4 at the 95% confidence level and were attributed to the emulsification technique where minute differences in the standard settling period can cause significant changes in the nominal fuel concentration.

The effect of renewing the contents of static bioassay vessels each 24 hrs during a 96-hr test (with the objective of attempting to compensate for the loss of volatile components) can be interpreted in different ways. For all of the materials tested, the 24-hr renewal of fuel throughout a 96-hr static bioassay produced a steadily decreasing LC 50 value (Figure 9). This was not so for all of the fuels examined without 24-hr renewals. Thus with MCH and the fuel JP4, which are (or contain) the most volatile components studied in static bioassay of emulsions, the full toxic effect observed after 96-hr exposure was virtually all exerted in the first 24 hrs. This observation could support the hypothesis that an initially toxic component is being lost from the assay solution, possibly by stripping or volatilization. However one must also be cognizant of the fact, illustrated by Figure 9, that in only one instance (that of RJ4) was the 96-hr LC 50 significantly different between renewed and non-renewed assays. This does not support the hypothesis that a toxic-volatile component is being lost from the assay vessels; however, for the two most volatile fuels (JP4 and MCH) the early (24-, 48-hr) values of LC 50 are significantly higher for the renewed fuels as compared to the non-renewed fuels. It is therefore possible that these results are confounded by differences in toxicity due to variability in emulsion preparation.

A comparison of the toxicity of JP9 with the sum of the toxicities of its components was made using the Toxic Unit method of Sprague (1969). The toxic units of the JP9 components were summed in direct proportion to the weight composition of each component in JP9, i.e.

$$JP9_{TU} = 0.15\ RJ4_{TU} + 0.45\ RJ5_{TU} + 0.4\ MCH_{TU} \quad .$$

The toxicity computed by this method is in good agreement with the experimentally-measured toxicity of JP9 for all experiments of up to 48-hr duration (Figure 10). For experiments where there was no fuel renewal throughout the 96-hr bioassay period, the computed and measured JP9 toxicities were in excellent agreement. For bioassays in which the fuel components were renewed each 24 hrs, the sum of the component toxicities predicted a

TABLE 4

FUEL AND FUEL COMPONENT EMULSION TOXICITY IN STATIC BIOASSAYS — TEST ORGANISM, GOLDEN SHINER

Fuel or Fuel Component	Renewal Time, hr	Total Hardness, mg $CaCO_3/\ell$	LC 50, $\mu\ell/\ell$				95% Confidence Limits of 96-hr LC 50, $\mu\ell/\ell$
			24 hr	48 hr	72 hr	96 hr	
RJ 4	96	123	5.0	2.6	1.9	1.7	1.3 - 2.4
	96	38	4.4	2.5	2.0	1.7	0.94 - 3.2
	24	123	3.6	0.95	0.56	0.51	0.35 - 0.75
RJ 5	96	123	1.9	1.5	1.1	1.0	0.73 - 1.4
	96	38	3.2	1.3	1.2	1.0	0.68 - 1.5
	24	123	6.0	1.6	0.8	0.61	0.39 - 0.96
MCH	96	123	53	53	53	53	33 - 85
	96	38	81	81	81	81	63 - 104
	24	123	--	126	90	72	56 - 95
JP 9	96	123	8.8	2.5	2.1	1.9	1.1 - 3.2
	24	38	10	3.7	2.7	2.0	1.3 - 2.9
JP 4	96	123	50	49	49	49	38 - 63
	96	38	16	16	16	16	8 - 31
	96	38	20	19	18	18	9 - 31
	24	123	48	28	22	17	11 - 26
	24	38	66	33	27	19	13 - 27

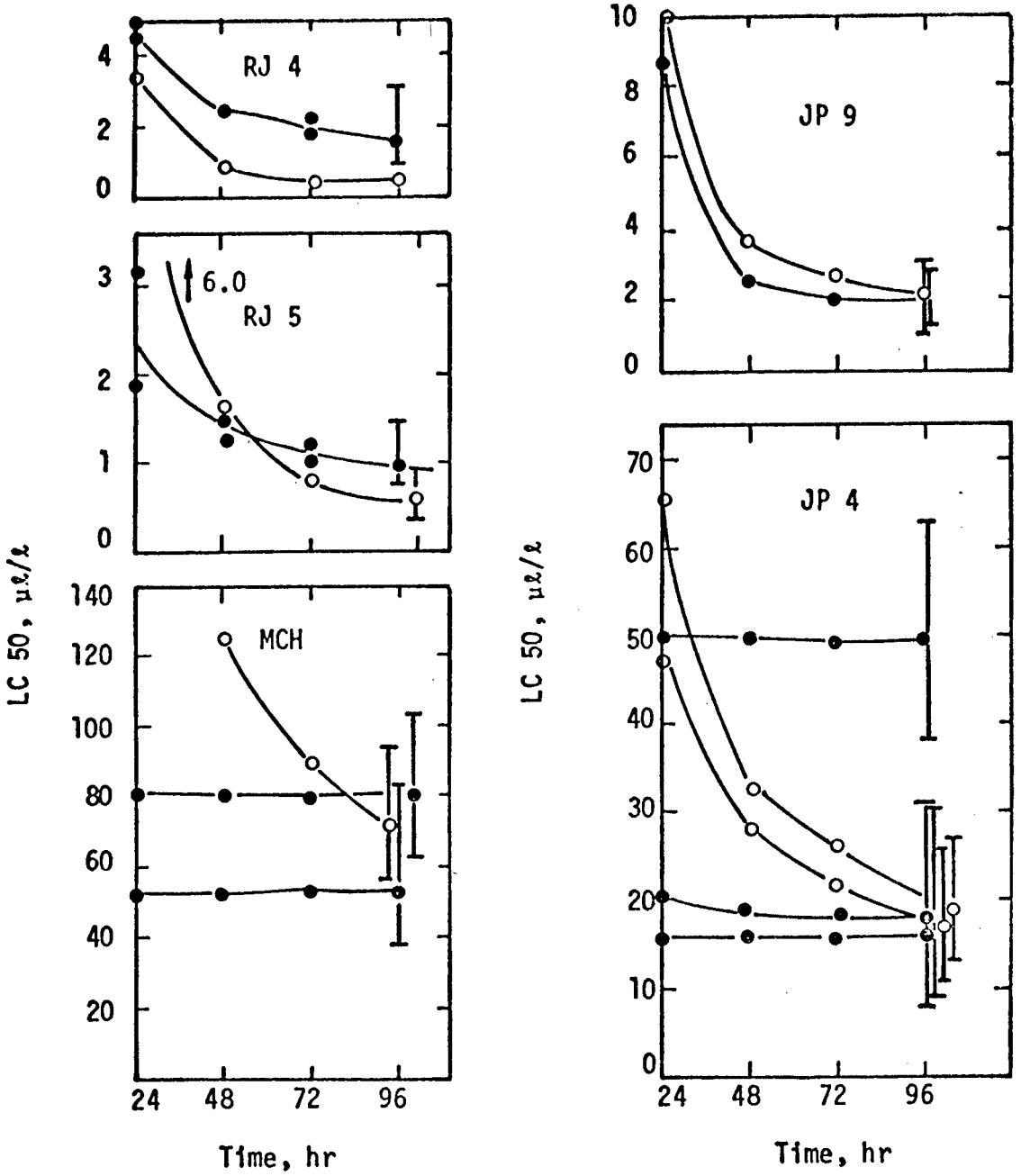


FIGURE 9. FUEL AND FUEL COMPONENT EMULSION TOXICITY IN STATIC BIOASSAYS. TEST ORGANISM, GOLDEN SHINER

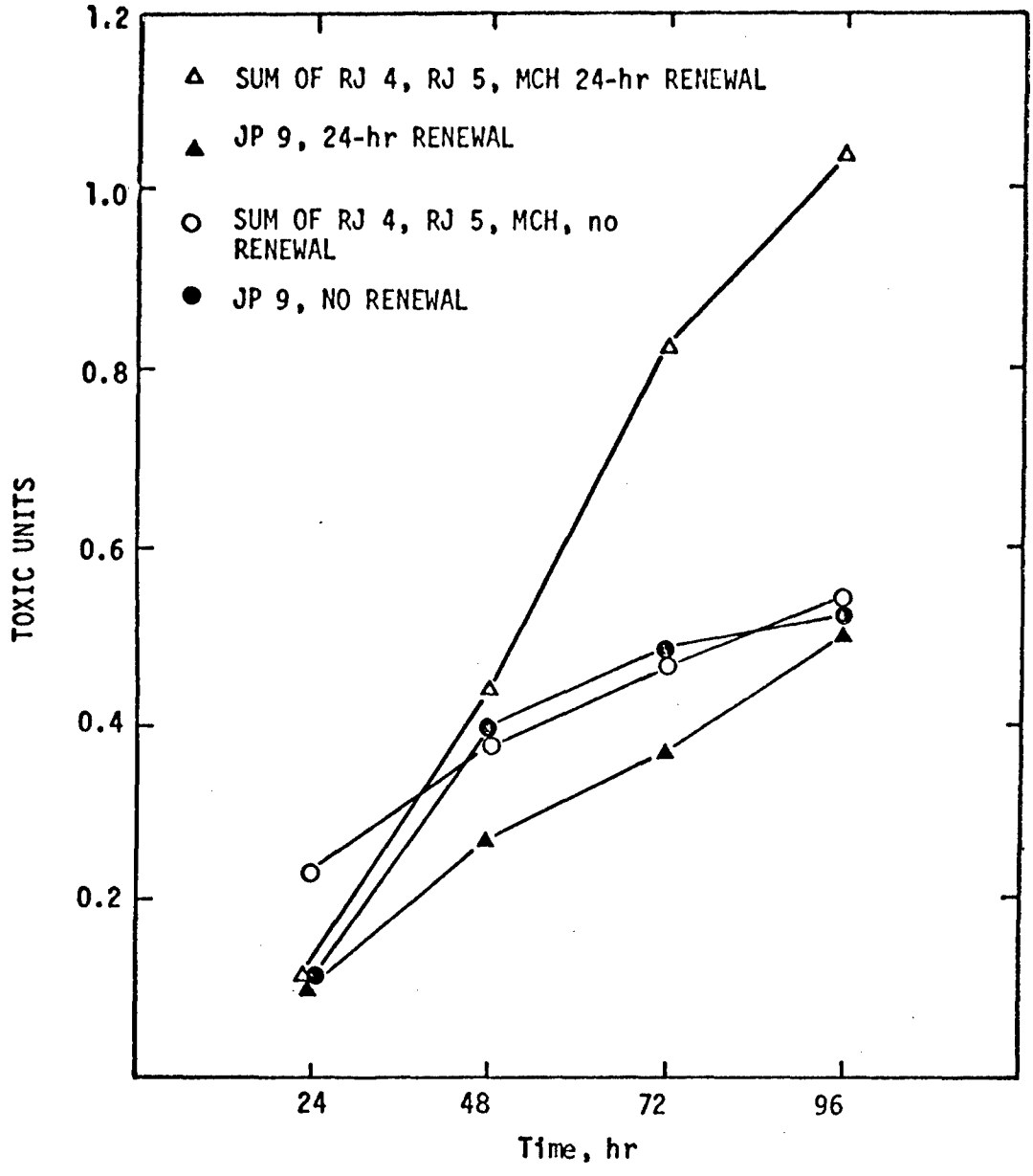


FIGURE 10. COMPUTED AND OBSERVED TOXICITY OF JP 9 FOR 24-hr RENEWAL AND NONRENEWAL EMULSION STATIC BIOASSAYS

progressively higher toxicity for JP9 than was observed at 72 hrs and at 96 hrs.

These results are consistent with the premise that one mode of exposure is physical contact of the fish with droplets of the emulsified fuel. When fuel is not renewed during the 96-hr bioassay, the emulsion is given time to separate so that physical contact is less important than intoxication from dissolved components. In this situation, computed and observed toxicities are in excellent agreement.

When the fuel and fuel components are renewed each 24 hrs throughout the 96-hr assay period, the emulsion is not given an opportunity to separate. In this instance physical contact between the fish and fuel droplets is more important. When one sums the toxicities of the three JP9 components, the effect of physical contact is counted three times because the component bioassays were conducted in separate vessels. The experimental measurement of JP9 emulsion toxicity however only includes the effect of physical contact once. Thus, for stable emulsions the prediction of toxicity of JP9 from its components' toxicities should give larger values than measured experimentally for JP9 and these values should become more divergent with the duration of the experiment. The results are in excellent agreement with this hypothesis and show that for the stable emulsions the toxicity of JP9 can only be predicted from its components' toxicities up to 48 hrs. Beyond that time the component toxicities overpredict JP9 toxicity.

Fish weight and length measurement (Table 5) indicate that smaller (and therefore more sensitive) fish were used in assay vessels in which the fuel was not renewed as compared to those used in the vessels in which fuel was renewed each 24 hrs. In general, the fish weight during non-renewal studies was approximately 0.8 g or one-half the 1.6 g mean weight of fish available for the renewal studies. Thus, the increased toxic response to fuels indicated in the renewal studies occurred despite the larger fish used, and suggests that perhaps toxicity would be even greater were the smaller fish utilized when solutions were renewed.

Non-Emulsified Fuels—Static assays, in which Golden Shiners were placed in water in contact with floating or bottom pools of fuel and fuel components, indicated lower toxicity than the respective emulsified fuel in all instances (Table 6). Using the 96-hr LC 50 for comparative purposes, MCH was 3 times less toxic, JP4 was 32 times less toxic, RJ4 was 100 times less toxic and RJ5 was 4,700 times less toxic than the corresponding emulsified materials. Beside this decrease in toxicity, it is interesting to note that RJ4 and RJ5 toxicities were widely different (96-hr LC 50 values of 100 µℓ/ℓ and 4,700 µℓ/ℓ, respectively) while they were virtually of identical toxicity in the emulsified form (0.51-1.7 µℓ/ℓ and 0.61-1 µℓ/ℓ, respectively). It is evident from these data that it is possible to have a wide range of toxicity exerted by a single fuel component depending on its physical state. Moreover, from the amount of data available it does not appear to be possible to predict the relative toxicities of two fuel components in one physical state from a knowledge of their toxicity in another physical state.

Table 5

FISH STATISTICS IN EMULSION STUDIES

Toxicant	Renewal Time hr	Mean Fish Weight g	Mean Fish Length	
			cm	±s
RJ4	96	0.85	4.07	0.14
RJ4	96	0.73	3.89	0.17
RJ4	24	1.6	4.71	0.40
RJ5	96	0.85	4.07	0.14
RJ5	96	0.73	3.89	0.17
RJ5	24	1.6	4.71	0.40
MCH	96	1.6	4.45	0.27
MCH	96	1.6	4.71	0.40
MCH	24	1.6	4.71	0.40
JP9	96	1.6	4.45	0.27
JP9	24	1.6	4.71	0.40
JP4	96	0.85	4.07	0.14
JP4	96	0.73	--	--
JP4	96	--	--	--
JP4	24	1.5	4.45	0.27
JP5	24	1.6	4.71	0.40

Table 6

FUEL AND FUEL COMPONENT TOXICITY IN STATIC BIOASSAY
USING PURE FUEL OR FUEL COMPONENTS—TEST ORGANISM: GOLDEN SHINER

Toxicant	LC 50, µℓ/ℓ Time in Hours				95% Confidence Limits 96-hr LC 50 µℓ/ℓ	Mean Fish Weight g	Average Standard Length cm
	24	48	72	96			
RJ4	--	5000	100	100	--	0.86	4.17±0.22
RJ5	--	8000	6100	4700	3200 - 7100	0.87	4.17±0.22
MCH	240	240	240	240	190 - 300	1.60	4.45±0.27
JP9	560	490	490	470	340 - 640	1.50	4.45±0.27
JP4	1600	620	570	570	400 - 800	0.87	4.17±0.22

There is considerable evidence that one important route of exposure in the non-emulsified fuel bioassays was by direct contact of the fish with the fuel on the surface of the water. Thus for RJ4 the percent survival of test fish was a function of time of exposure rather than the volumetric concentration of the fuel (Figure 11). Evidence exists also in the pattern of fish survival for RJ5 that physical contact between fuel and fish is involved in the toxic response. However, since this component is more water soluble than RJ4, the response due to physical contact is not as unequivocal as with RJ4. For MCH, which is both more soluble than RJ4 and RJ5 as well as being more volatile (Table 1), the toxicity response is typical for a dissolved toxicant (Figure 11). However, since the toxic response is identical at 24, 48, 72 and 96 hrs, one must assume that any toxic effect is exerted immediately. This type of response would be expected of a highly volatile toxicant which was stripped out of the assay vessel rapidly. The response of fish to JP9 is typical of a response to a soluble toxicant.

Further evidence in support of the direct contact mode of exposure to pure fuels is provided by the relatively low toxicity of water-soluble fractions of MCH and JP4. The 96-hr LC 50 values (Table 7) show that water-soluble MCH was some 979 times less toxic than pure MCH in contact with water while the water-soluble components of JP4 were some 351 times less toxic than the pure fuel in contact with water.

Table 7

WATER-SOLUBLE FUEL AND FUEL COMPONENT TOXICITY
IN STATIC BIOASSAYS—TEST ORGANISM GOLDEN SHINER

Fuel or Fuel Component	LC 50, µℓ/ℓ				95% Confidence Limit of 96-hr LC 50 µℓ/ℓ
	24 hr	48 hr	72 hr	96 hr	
MCH	309,000	243,000	239,000	235,000	188,000 - 295,000
JP4	313,000	267,000	212,000	200,000	174,000 - 231,000

Discussion

Static bioassays revealed a number of significant findings concerning the two basic fuels, JP4 and JP9, and pertinent phenomena relating to the behavior of the formulated JP9 when it is added to water. Visual observation indicated that during the course of the 96-hr tests with JP9 there was a

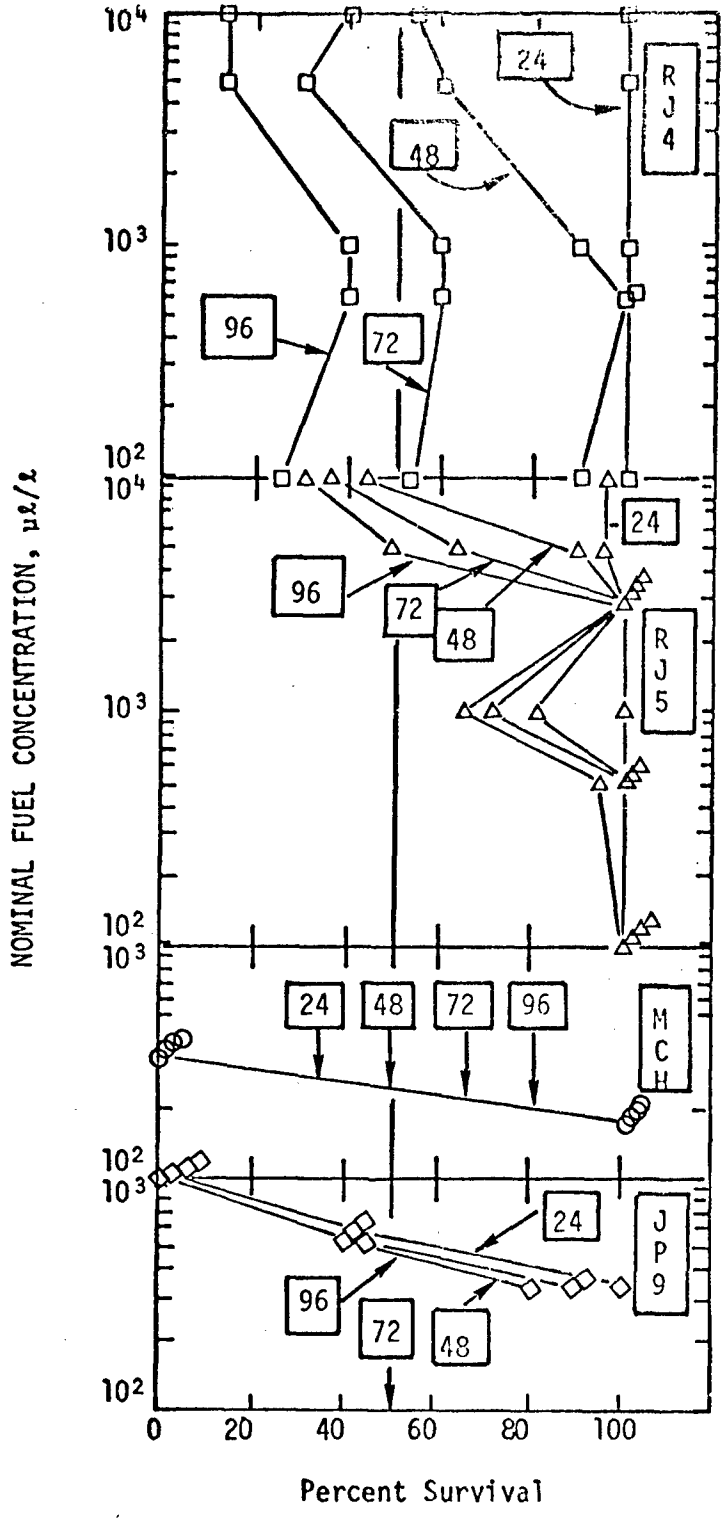


FIGURE 11. RELATIONSHIP OF FISH SURVIVAL TO EXPOSURE TIME AND TOXICANT CONCENTRATION FOR JP 9 AND ITS COMPONENTS RJ 4, RJ 5 AND MCH

continual buildup of RJ5 droplets on the bottom of assay vessels. These phenomena suggested that there was a progressive losss by volatilization of the solubilizing agent, MCH, which caused the dissassociation of RJ5.

Although less evident it is presumed that the RJ4 fraction of JP9 also tends to disassociate and rise to the surface. This disassociation lends added importance to a consideration of what happens to the various components of JP9 in independent analyses. Thus, the fate of these components, their individual toxicities to aquatic life, the mode of exposure, and the mechanism of toxicity must be evaluated together with the formulated fuel, JP9, and JP4.

The results show that the mode of fuel exposure to fish is of overriding importance in assessing the relative toxicities of the fuels and components under investigation. In the emulsified form, RJ4 and RJ5 are the most toxic of the materials studied with a 96-hr LC 50 of approximately 1.0 µℓ/ℓ (and about 0.5 µℓ/ℓ if 24-hr solution renewal is used). However, in the non-emulsified, or pure fuel form, RJ5 is by far the least toxic of the materials studied exhibiting a 96-hr LC 50 of 4700 µℓ/ℓ. This vast difference in toxicity between the two modes of exposure is indicative of the relative insolubility of RJ5, and also of the limited opportunity for contact with fish to the non-dispersed pure fuel which resides on the bottom. The fact emerges from the emulsified fuel studies that RJ5 is extremely toxic to fish when it is dispersed, enabling the opportunity for contact.

Both RJ5 and RJ4 show an unusual pattern of toxicity in the pure fuel form (Figure 11), demonstrating a time response rather than a concentration response. Over an extremely wide range of concentrations (100 to 10,000 µℓ/ℓ), their toxicity is virtually the same, suggesting that toxicity was due to chance encounters of fish with fuel globules rather than to contact with the soluble fraction of the fuels. With the progression of time, the probability of an actual physical contact between fish and the fuel globule increases. Because Golden Shiners are not bottom feeders, they are more likely to come into contact with RJ4 globules than RJ5 globules. For bottom-feeders, it is likely that the reverse would be true, and RJ5 would be the most toxic component when present in a droplet form.

Comparing the two fuels, JP4 and JP9, the following facts emerged. In the emulsified form, JP9 is approximately 9 times more toxic than JP4 to Golden Shiners. The relative 96-hr LC 50's of the two materials are 2.0 µℓ/ℓ for JP9 and about 18 µℓ/ℓ for JP4.

In the non-emulsified (pure fuel) form, JP9 is slightly more toxic to Golden Shiners than JP4 at 470 µℓ/ℓ (96-hr LC 50). While the 95% confidence limits indicate that this difference is not significant, it should be noted that the fish size was larger for the JP9 study (mean weight of 1.5 g compared to 0.87 g) and this could have reduced the sensitivity of fish to JP9.

IV. CONTINUOUS-FLOW FLAGFISH BIOASSAY

Introduction

Continuous-flow bioassays were conducted on the water-soluble fractions of fuels and fuel components to determine egg hatchability and fry development effects for the flagfish (Jordanella floridae) in the presence of MCH, RJ4, RJ5 and JP9 (containing weight percentages of 16.2% RJ4, 56.8% RJ5 and 27% MCH). Flagfish eggs, fertilized by one male, were transferred from spawning aquaria to egg cups in the continuous-flow exposure tanks. After hatching (approximately one week), young fry were transferred to fry chambers located about 1 ft from the inlet end of the exposure tank. After about 1 month, the fry had grown sufficiently to permit their release into the effluent-end chamber of the exposure tanks where they remained for the duration of the experiment.

Results

Environmental Conditions—Periodic water analyses (Table 8) showed the water of low TDS and hardness. Exposure tank temperature was maintained at 25°C (±0.5°C), dissolved oxygen at >7 mg/ℓ and pH at 8.0.

Table 8

RANGE OF WATER CHARACTERISTICS FOR FLAGFISH EGG HATCHABILITY AND FRY DEVELOPMENT STUDY

Analysis	Concentration Range
pH	7.4 - 7.5
Alkalinity	40 - 47 mg $CaCO_3$/ℓ
Hardness	24 - 30 mg $CaCO_3$/ℓ
Ca	20 - 24 mg $CaCO_3$/ℓ
Mg	4 - 6 mg $CaCO_3$/ℓ
Cl	20 - 47 mg/ℓ
SO_4	1 - 2.1 mg/ℓ
Na	12 mg/ℓ
K	0.5 mg/ℓ
TDS	67 - 120 mg/ℓ

Fuel Concentrations in Fish Tanks—During the experiments, fuel concentrations were measured periodically at two locations in the dosing apparatus—the final head tank (HT) and the diluter effluent (DE) and in each fish tank. It was observed that droplets of pure fuel were being carried over from the contact and dilution apparatus into the fish tanks. This carry-over interfered with the conduct of the experiments on RJ4, RJ5 and JP9. The MCH exposure tanks were not notably affected by this problem because of the low specific gravity of MCH. Thus, as MCH droplets were carried over from the fuel contact chamber, they tended to rise to the water surface and to be expelled through the overflow of the final head tank into the drain; they did not normally travel through the proportional diluters into the fish tanks.

The data for MCH (Table 9 and Figure 12) are illustrative of two problems encountered with the use of the Mount-Brungs (1969) dilution device for poorly water-soluble, volatile compounds. Significant volatilization of MCH took place between the final head tank of the contacting device and the effluent from the diluter (the influent to the "100% Exposure Tank"). It is also evident from Table 9 that there was considerable temporal variation in the exposure tank MCH concentrations. Such variation is attributable largely to inconsistencies in the dosing and dilution apparatus because the MCH analytical technique has a coefficient of variation of only 8%. It is noteworthy that the MCH concentration in the exposure tanks did not decrease linearly with nominal volumetric dilution (Figure 12).

Table 9

MCH CONCENTRATIONS IN DILUTION APPARATUS
AND EXPOSURE TANKS DURING CONTINUOUS-FLOW BIOASSAY ON FLAGFISH

Sample Location	No. of Analyses	Mean MCH Concentration, mg/ℓ	Standard Deviation mg/ℓ	Coefficient of Variation, %
Head Tank	6	3.41	2.22	65
Diluter Effluent	7	1.85	0.82	44
100% Exposure Tank	8	0.83	0.73	88
50% Exposure Tank	6	0.67	0.65	97
12% Exposure Tank	6	0.34	0.38	112
Control Exposure Tank	6	0.01	0.01	100

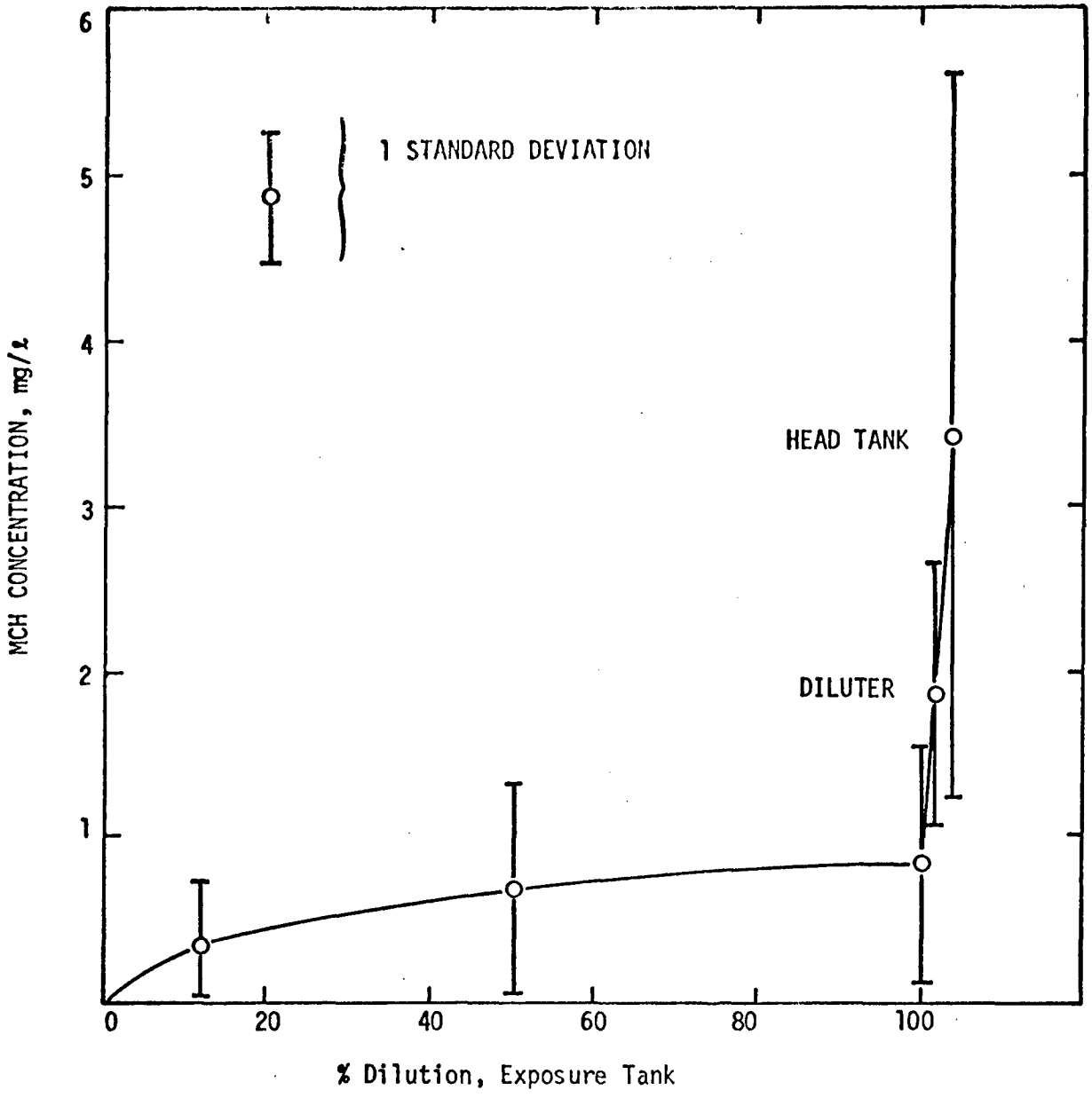


FIGURE 12. MCH CONCENTRATION VARIATION IN DILUTION APPARATUS AND EXPOSURE TANKS DURING CONTINUOUS FLOW BIOASSAY ON FLAGFISH

Further, though less well-defined data exists for RJ4, RJ5 and JP9 (Table 10), these data substantiate the problems with volatility losses in the dilution apparatus and for JP9 show that the volatility losses affect the relative composition of the fuel. Thus, the fractions of RJ4 and RJ5 in JP9 fall respectively from 14% and 56% in the head tank to 6% and 24% in the "12% Exposure Tank" while the MCH increases from 30% to 71% of the JP9.

Egg Hatchability—A minimum of 92, 1-4 day old eggs were placed in egg cups in each dilution. The egg cups were located adjacent to the outlet of a recirculation pump so that they were agitated by the flow from the pump. The eggs generally hatched within one week following immersion in the exposure tanks.

Water-soluble fractions of MCH and RJ4 had no significant effect on egg hatchability up to average concentrations of 0.83 mg/ℓ MCH and 0.2 mg/ℓ RJ4 (Figure 13). It appeared that RJ5 at concentrations of greater than 0.05 mg/ℓ significantly reduced flagfish egg hatchability. The water-soluble components of JP9 at an average concentration of 0.23 mg/ℓ appeared to reduce flagfish egg hatchability. The data presented in Figure 13 would support the view that the effect of JP9 on egg hatchability in this experiment was due to the toxicity of its RJ5 component. However, because of the variability in the hatchability of the controls and in the concentrations of fuels and fuel components in the various exposure tanks, this conclusion must be regarded as tentative.

Effect of MCH on Flagfish Fry Development—Because of the previously-mentioned problem of fuel droplet carryover, it was only possible to conduct fry development studies on MCH. Fry were kept in fry-chambers for 37 days following hatching then released to the exposure tanks for the remainder of the 87-day exposure period. No significant toxic effects as assessed by fish deaths and by measurement of fish lengths and weights following exposure were observed at any dilution of the water-soluble components of MCH (Figure 14). Reference to Figure 14 will show that while it was not possible to obtain standard deviations for fish weights (because the fish were weighed in batches rather than individually), the average wet weight of fish in all MCH concentrations up to 0.83 mg/ℓ was within the band of values encompassed by the average wet weights for the control exposure tanks.

These data indicate that average aqueous concentrations of 0.83 mg/ℓ MCH or less are not toxic to flagfish in the egg or fry development stages. Because of the loss of MCH between the contact/dilution apparatus in the exposure tanks, it was decided to conduct a static bioassay test on five flagfish using the diluter effluent (inlet to the "100% Exposure Tank"). In this solution there was an average concentration of 1.85 mg/ℓ MCH and all of the fish died after seven days exposure. These results allow one to tentatively set the acute toxic level for MCH aqueous solutions between 0.83 mg/ℓ and 1.85 mg/ℓ.

Effect of RJ4 on Flagfish Fry Development—The results obtained from these studies were confounded by fuel carryover. Mechanical problems resulted in an initial high rate of fuel carryover and all fry in the "100%" and "50%" exposure tanks died after 13 days; in addition, some

TABLE 10

AVERAGE CONCENTRATIONS OF SOLUBLE JP 9 COMPONENTS IN CONTINUOUS FLAGFISH BIOASSAY

Fuel or Fuel Component	Component Concentration, ±1 Standard Deviation, mg/ℓ						
	Head Tank	Diluter Effluent	100% Exposure Tank	50% Exposure Tank	25% Exposure Tank	12% Exposure Tank	Control Exposure Tank
MCH	3.41 ± 2.22	1.85 ± 0.82	0.83 ± 0.73	0.67 ± 0.65	--	0.34 ± 0.38	0.01 ± 0.01
RJ 4	13.9 ± 3.68	2.36 ± 2.22	0.20 ± 0.03	0.19 ± 0.05	0.15 ± 0.05	0.089 ± 0.04	0.001 ± 0.002
RJ 5	0.83 ± 0.89	0.32 ± 0.35	0.11 ± 0.09	0.12 ± 0.13	0.067 ± 0.07	0.043 ± 0.05	0.045 ± 0.06
JP 9	7.15 ± 5.53	1.39 ± 0.92	0.23 ± 0.11	0.17 ± 0.13	0.11 ± 0.07	0.15 ± 0.12	0.01 ± 0.01
MCH	2.15 ± 2.15	0.59 ± 0.37	0.13 ± 0.09	0.11 ± 0.05	0.05 ± 0.04	0.11 ± 0.09	0
RJ 4	1.00 ± 0.82	0.13 ± 0.13	0.01 ± 0.01	0.01 ± 0.007	0.02 ± 0	0.01 ± 0.007	0
RJ 5	4.00 ± 2.77	0.67 ± 0.81	0.09 ± 0.05	0.07 ± 0.06	0.05 ± 0.04	0.04 ± 0.04	0.01 ± 0.01

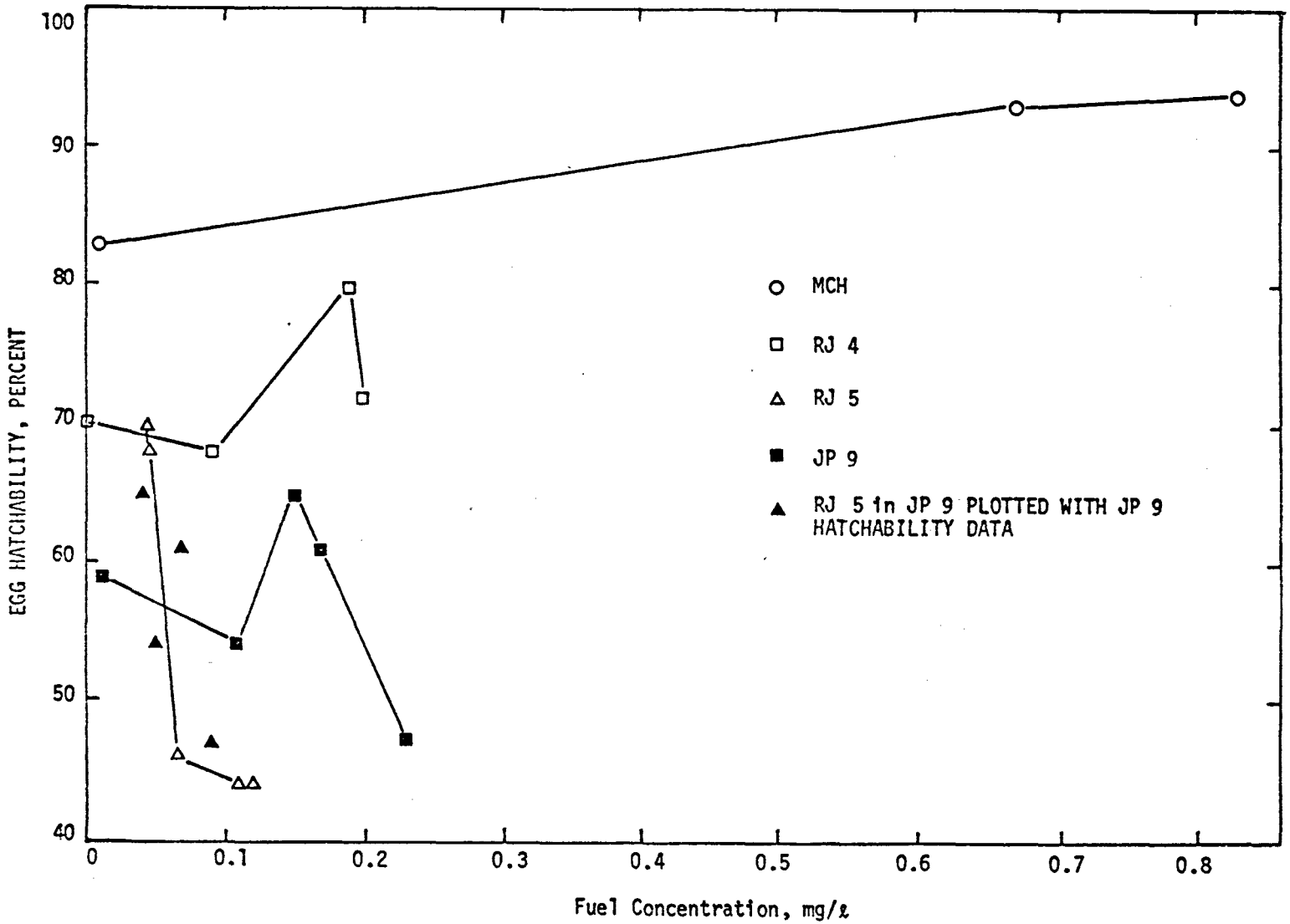


FIGURE 13. FLAGFISH EGG HATCHABILITY IN PRESENCE OF WATER SOLUBLE COMPONENTS OF FUELS AND FUEL CONSTITUENTS

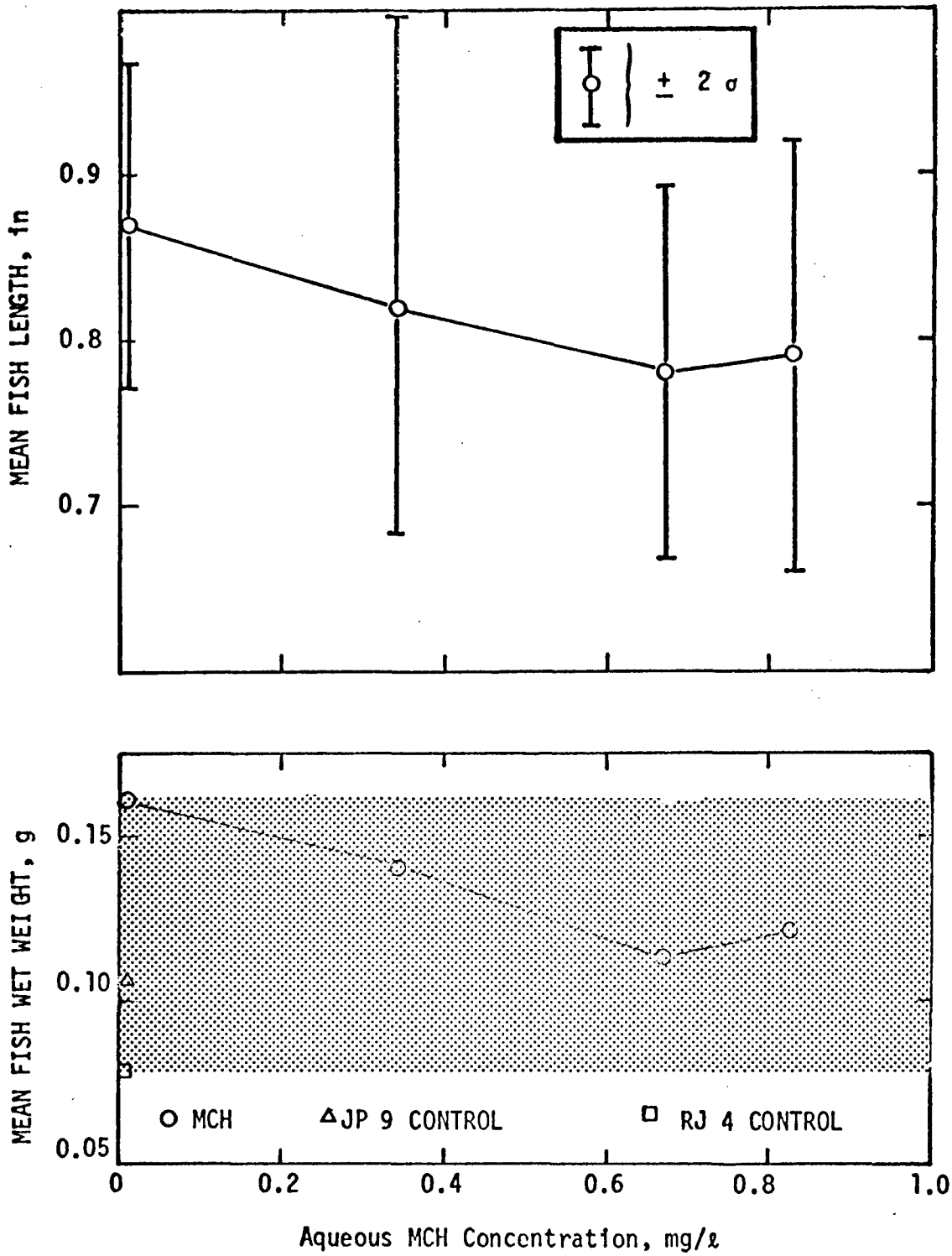


FIGURE 14. RELATIONSHIP OF FLAGFISH WET WEIGHT AND LENGTH TO AQUEOUS MCH CONCENTRATION IN CONTINUOUS BIOASSAY

20-50% of the fry in the other three exposure tanks were lost. The tanks were restocked with 42 fish each and in the ensuing 3 weeks, all fish died in the tanks containing mean RJ4 concentrations of 0.2 and 0.19 mg/ℓ ("100%" and "50%" tanks). In the exposure tank containing a mean RJ4 level of 0.14 mg/ℓ, 15 fish survived and in the presence of 0.089 mg/ℓ RJ4, 33 fish survived for the 57-day duration of the experiment—an identical number to that surviving in the control. It is possible to conclude that 0.089 mg RJ4/ℓ is non-lethal in an acute fashion but that 0.14 mg RJ4/ℓ causes some acute lethal response.

The lengths and weights of all surviving fish were measured (Table 11). The 15 survivors in the presence of 0.15 mg/ℓ RJ4 were significantly larger than those in the 0.089 mg/ℓ RJ4 or control tanks. It is possible that this is because the weaker, smaller fish were killed by 0.14 mg/ℓ RJ4, allowing the stronger and larger fish to survive. A possibility exists, however, that the presence of fewer fish in the 0.14 mg/ℓ RJ4 tank resulted in reduced competition for food and consequently larger fish (even though every effort was made to supply ample food).

Table 11

EFFECT OF RJ4 ON FLAGFISH LENGTH AND WEIGHT DURING 44 DAYS CONTINUOUS EXPOSURE

Exposure Tank	RJ4 Concentration mg/ℓ	Number of Survivors	Mean Length in	Standard Deviation of Length in	Coefficient of Variation of Length %	Mean Wet Weight g
25%	0.140	15	0.717	0.048	6.7	0.0920
12%	0.089	33	0.651	0.085	13.0	0.0704
control	0.001	33	0.686	0.074	10.8	0.0789

Effect of RJ5 and JP9 on Flagfish Fry Development—In these studies, carryover of RJ5 to the exposure tanks was evident as small globules on the bottom of each tank. This phenomenon occurred in the JP9 tanks because the RJ5 component separated from the soluble JP9 after passing through the diluters. Mortality was high in both banks of tank, apparently due in part to the tendency of flagfish to nibble on RJ5 droplets. The result was that in the JP9 study only 4 fish survived in the "12%" tank (equivalent to

0.15 mg/ℓ JP9) compared to 29 survivors in the control tank. These four surviving fish had an average length of 0.957 in (s = 0.080 in, C_V = 8.4%) and an average wet weight of 0.2278 g, while the 29 fish in the control tank had a average length of 0.749 in (s = 0.086 in, C_V = 11.5%) and an average wet weight of 0.1062 g. Therefore, the only fish capable of surviving in the presence of JP9 were unusually large.

By the end of the RJ5 studies (51 days), no fish survived in any of the tanks except the control (53 survivors). At the 40-day mark of the experiment, there were 5 survivors in the "12%" tank that contained an average RJ5 concentration of 0.043 mg/ℓ.

MCH Accumulation by Flagfish—At the conclusion of the 87-day MCH bioassay, live flagfish were removed from the exposure tanks and analyzed for MCH. Fish from a short-term (7-day) static bioassay conducted on MCH dilution tank effluent were also analyzed for MCH. The latter fish had been exposed to an average aqueous MCH concentration of 1.85 mg/ℓ and were all dead. The results (Figure 15) show that there is an almost linear relationship between total MCH body burden and aqueous MCH concentration and that flagfish can tolerate a total body burden of about 0.5 mg MCH/g wet weight for flagfish. Figure 15 also includes one data point from a rainbow trout experiment relating the total body burden of MCH to aqueous MCH concentration after 42 days exposure. It can be seen that there is excellent agreement between this data point and the flagfish data.

RJ4 Accumulation by Flagfish—The fish that died during the RJ4 bioassay as well as the fish surviving at the conclusion of the study were analyzed for RJ4. With three exceptions, samples of 15 or more fish were analyzed. Because fish were removed from exposure tanks directly after death, exposure times vary from 20 to 57 days (Table 12). The level of RJ4 accumulated by flagfish is a function of aqueous RJ4 concentration for surviving fish but not for dead fish (Figure 16). This result, together with the evidence that surviving fish can tolerate much higher body burdens of RJ4 than observed in the dead fish, suggests that the fish may have died because of contact with fuel droplets rather than by accumulation of fuel from "solution."

Discussion

The egg hatchability studies revealed a toxic effect at average aqueous RJ5 concentrations of greater than 0.05 mg/ℓ and at average aqueous JP9 concentrations of 0.23 mg/ℓ. Whether toxicity was due solely to the soluble fraction of these materials is uncertain because of the carryover of fuel droplets. It is apparent, however, that the presence of this fuel and of fuel components can interfere with egg hatchability.

No lethal effects of MCH on flagfish fry at concentrations up to 0.83 mg/ℓ were observed, but in a short-term study, lethality occurred at 1.85 mg/ℓ MCH. Chronic exposure to MCH concentrations up to 0.83 mg/ℓ for a period of 87 days appeared to cause a significant decrease in fish size at successively higher concentrations of MCH, if the size comparisons were made solely between MCH exposure tanks and the MCH control tank. However, this

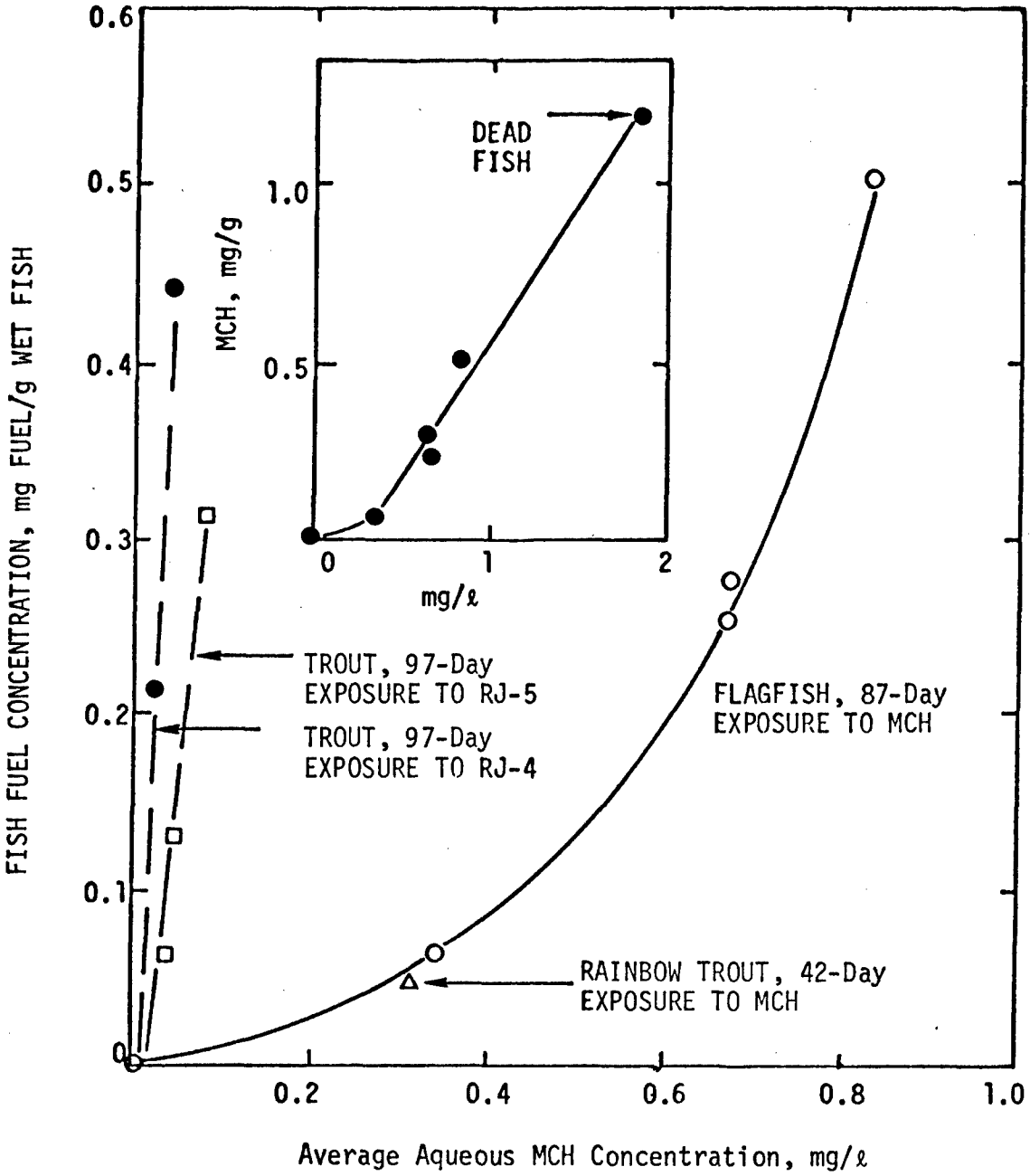


FIGURE 15. ACCUMULATION OF FUEL IN FLAGFISH AND TROUT AS A FUNCTION OF AQUEOUS FUEL CONCENTRATION

Table 12

RJ4 ACCUMULATION IN FLAGFISH

Exposure Tank %	RJ4 Concn mg/ℓ	Exposure Time Days	Fish			Fuel Accumulation $\frac{\text{mg RJ4}}{\text{g Fish}}$
			Number	Condition	Wet Weight g	
100	0.20	31	17	Dead	0.975	0.285
100	0.20	38	6	Dead	0.246	0.645
50	0.19	31	16	Dead	1.040	0.232
50	0.19	20	1	Dead	0.052	0.672
25	0.14	35	16	Dead	0.483	0.650
25	0.14	44	15	Alive	1.297	1.108
12	0.089	57	1	Dead	0.083	0.609
12	0.089	57	16	Alive	0.947	0.790
0	0.001	57	20	Alive	1.567	0.008

conclusion could not be supported when the fish exposed to MCH were compared with the entire control fish populations of each of the four banks of exposure tanks (for RJ4, RJ5 and JP9) run at the same time as the MCH experiment.

In the RJ4, RJ5 and JP9 bioassays interferences were caused by pure fuel carryover from the fuel contact and dilution device to the fish exposure tanks. Despite this complication, the flagfish survival rate at 0.089 mg/ℓ RJ4 matched the control survival, and there was approximately 50% fish mortality in 57 days exposure to 0.14 mg/ℓ RJ4. Only four fish survived exposure to 0.043 mg/ℓ RJ5 for 51 days, and no fish survived exposure to concentrations of JP9 in the range studied (0.11-0.23 mg/ℓ). It was noted that when a limited number of fish were present in an exposure tank, they often were much larger than the individuals of a more heavily populated tank. This could have reflected a natural selection process in which the larger individuals were those capable of surviving a stress situation. Another explanation might be the reduced competition for food among fewer individuals, although an effort was made to supply an excess of food during each feeding period so this type of competition would not be a factor.

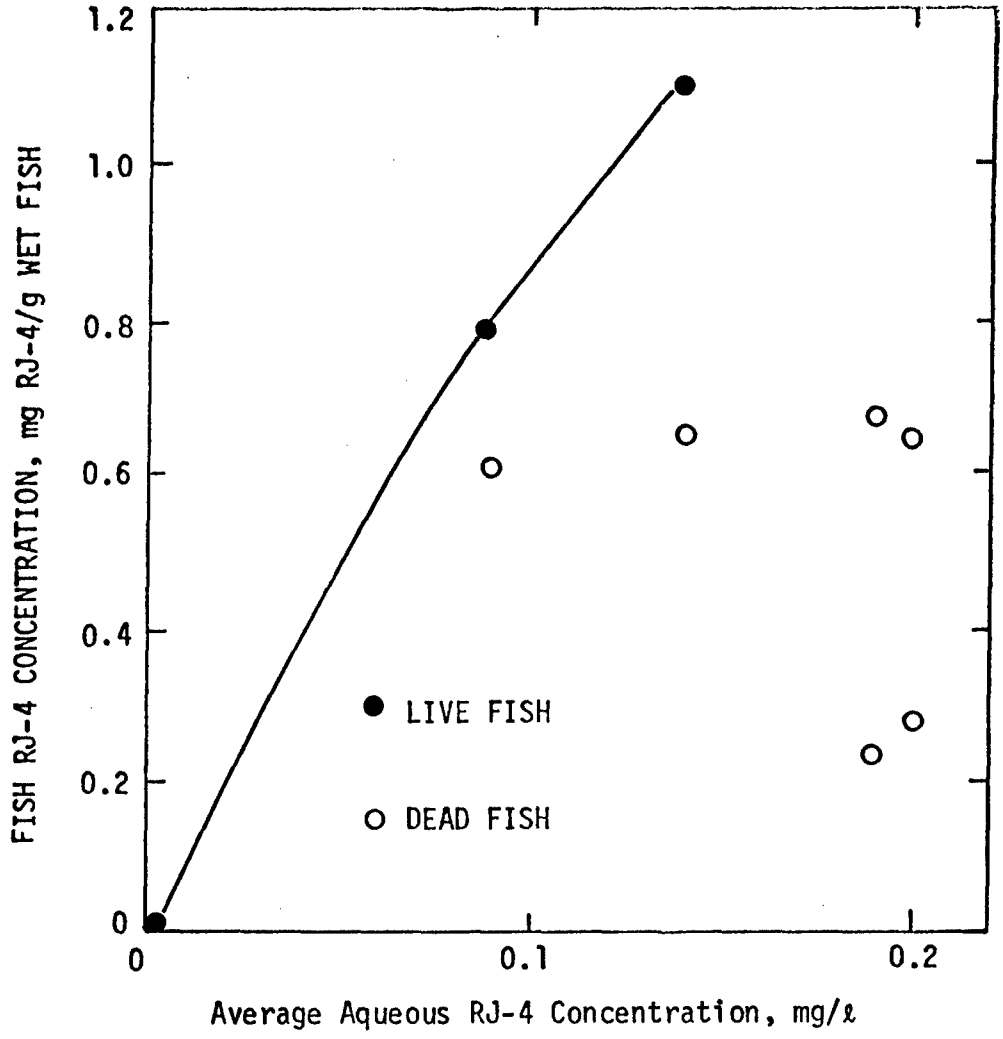


FIGURE 16. ACCUMULATION OF RJ-4 IN FLAGFISH AS A FUNCTION OF AQUEOUS RJ-4 CONCENTRATION (20-57 Day Exposure)

An almost linear relation existed between total MCH body burden and aqueous MCH concentration. A body burden of 0.5 mg MCH per g wet fish weight appeared to be tolerated without lethal effects over a 87-day period. It appeared that a body burden of 1.19 mg MCH per g wet weight could not be tolerated without death in a 7-day time interval.

Accumulation of RJ4 in flagfish also appeared to be linear with respect to aqueous RJ4 concentration for survivors to RJ4 exposure. Those fish that died during exposure did not indicate that accumulation of RJ4 was a function of aqueous concentration, indicating that death was possibly caused by random contact with fuel droplets.

V. CONTINUOUS-FLOW RAINBOW TROUT BIOASSAYS

Introduction

Prior to the commencement of the continuous trout bioassay of soluble components of fuels and fuel constituents, the fuel-contacting apparatus was modified to prevent the fuel droplet carryover that had caused problems in the previous flagfish assay. Synthetic wool was placed in the separation chamber (Figure 1) to reduce turbulence and to intercept fuel globules. Water flow rates were reduced in the fuel chambers to 1400 mℓ/min. Restraining screens were placed over the exposure tanks in an attempt to prevent fish from jumping out of the tanks. Even with these screens in place, some fish succeeded in jumping out of the tanks, especially at the higher fuel concentrations. Thirty fish were used in each exposure tank. A water temperature of 15°C was maintained during the assay.

Results

RJ4—In a 97-day exposure to various dilutions of an aqueous solution of RJ4, almost all observed deaths occurred during the first 23 days exposure (Table 13). At this time there were no deaths in the control tank (RJ4, 0.001 ±0.002 mg/ℓ), two deaths at an RJ4 concentration of 0.031±0.023 mg/ℓ, 18 deaths at 0.048±0.019 mg/ℓ RJ4, and no survivors (29 deaths) in the exposure tank containing an average RJ4 concentration of 0.068±0.025 mg/ℓ. There were no survivors (29 deaths) at an RJ4 level of 0.122±0.057 mg/ℓ. In the remaining 74 days of the experiment, only 2 further deaths occurred (one in the control tank and one in the tank containing 0.031±0.023 mg/ℓ RJ4). Non-lethal aqueous concentrations of RJ4 could not be established, but they are certainly below 0.03 mg/ℓ (Table 13). The LC 50 value for this exposure time was estimated to be 0.045 mg/ℓ (Figure 17).

RJ5—The RJ5 study lasted for 97 days. There were no deaths in the control exposure tank, 4 deaths each in the exposure tanks containing average RJ5 concentrations of 0.04 and 0.05 mg/ℓ; 8 deaths in the presence of 0.08 mg/ℓ RJ5 and no survivors in the presence of 0.12 mg/ℓ RJ5 (Table 14). All fish deaths occurred during the first 14 days of the experiment. Non-lethal RJ5 aqueous concentrations could not be established, but they are certainly below 0.04 mg/ℓ. The LC 50 value for this exposure time was estimated to be 0.072 mg/ℓ (Figure 17).

MCH—The experiments on both MCH and JP9 had to be terminated after 23 days because of a failure in the refrigeration equipment that allowed the temperature of the exposure tanks to rise to 20°C and caused significant mortalities. However, on the basis of the RJ4 and RJ5 experiments, it is likely that the deaths occurring during this period are representative of those that would have taken place over the longer (97-day) exposure time used in the RJ4 and RJ5 experiments. On this basis it would appear that the chronic non-lethal level for MCH is some value below 0.8 mg/ℓ. This value should be interpreted with caution because at an average concentration

Table 13

CUMULATIVE RAINBOW TROUT DEATH TALLY FOR CONTINUOUS-FLOW CHRONIC BIOASSAY ON WATER-SOLUBLE COMPONENTS OF RJ4

Exposure Time Days	Cumulative Death Tally, Numbers of Fish				
1	0	0	0	0	0
3	0	0	0	8	23
4	0	1	5	16	28
5	0	1	6	22	28
6	0	1	8	22	29
10	0	2	10	28	29
14	0	2	13	28	29
23	0	2	18	29	29
34	1	2	18	29	29
35	1	3	18	29	29
57	1	3	18	29	29
77	1	3	18	29	29
97	1	3	18	29	29
Water Soluble RJ 4 Concentration, mg/ℓ	0.001	0.031	0.048	0.068	0.122
Standard Deviation, mg/ℓ	0.002	0.023	0.019	0.025	0.057

TABLE 14

SURVIVAL OF RAINBOW TROUT IN CONTINUOUS FLOW BIOASSAY OF WATER SOLUBLE COMPONENTS OF RJ 4, RJ 5, MCH AND JP 9

RJ 4			RJ 5			MCH		JP 9	
Average Concentration mg/ℓ	Survival % 97 Days Exposure	Survival % 23 Days Exposure	Average Concentration mg/ℓ	Survival % 97 Days Exposure	Survival % 23 Days Exposure	Average Concentration mg/ℓ	Survival % 23 Days Exposure	Average Concentration mg/ℓ	Survival % 23 Days Exposure
0.001	97	100	0	100	100	0.01	97	0.08	100
0.031	90	93	0.04	87	87	0.31	97	0.12	97
0.048	40	40	0.05	87	87	0.80	97	0.26	97
0.068	0	0	0.08	73	73	0.84	77	0.37	57
0.122	0	0	0.12	0	0	1.19	60	0.60	0

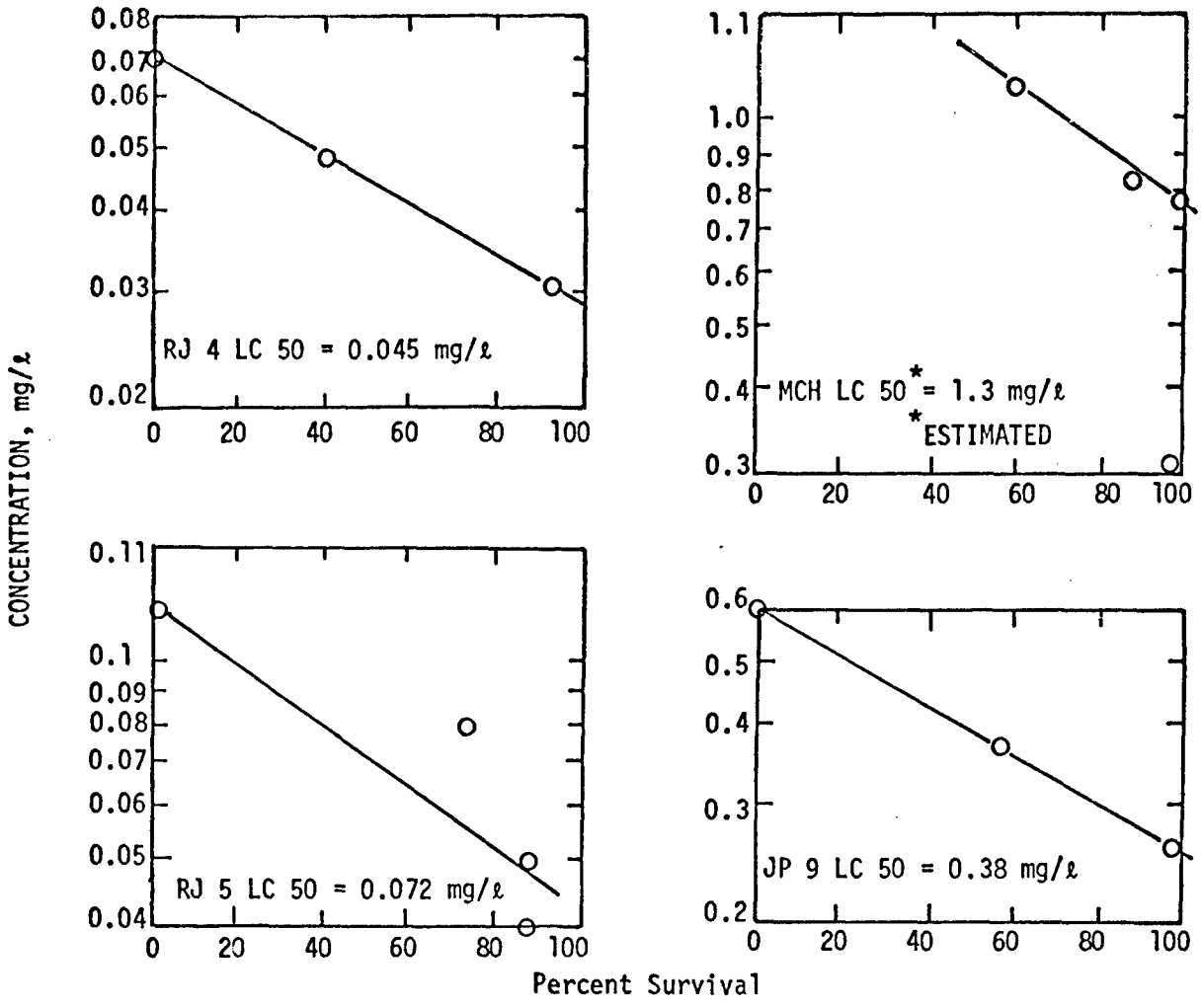


FIGURE 17. 23-DAY LC 50 VALUES FOR RAINBOW TROUT IN CONTINUOUS FLOW BIOASSAYS

used only caused 40% mortality. However, from the data obtained, a 23-day LC 50 value of 1.3 mg/ℓ is estimated.

JP9—The JP9 experiment terminated after 23 days because of temperature control problems. The experimental data indicate a non-lethal concentration of some value less than 0.37 mg/ℓ and a 23-day LC 50 value of 0.38 mg/ℓ. In reporting these values, one must be aware that the aqueous solutions of JP9 in the fish exposure tanks are grossly different in composition from the JP9 fuel used to make up the aqueous JP9 solution. This is because of both differential solubility and volatility of JP9 components. Thus the neat JP9 fuel contained some 27% MCH, 16% RJ4 and 57% RJ5 while the aqueous JP9 solutions in the exposure tanks had an average composition of 85.4% MCH, 1.8% RJ4 and 13.3% RJ5. Using the toxic unit summation method of Sprague (1969) to predict the toxicity of JP9 from its components RJ4, RJ5 and MCH, gave excellent results when based on the JP9 composition found in the exposure tanks (Figure 18). However, if these computations had been based on the original fuel composition, the prediction of toxicty would have been grossly in error (Table 15).

Table 15

PREDICTED AND OBSERVED TOXICITY OF WATER-SOLUBLE JP9 COMPONENTS TO RAINBOW TROUT IN 23-DAY CONTINUOUS BIOASSAY

JP 9 "Concentration" mg/ℓ	Toxic Units Observed	Toxic Units Predicted	
		Based on Exposure Tank JP 9 Composition	Based on JP 9 Fuel Feed Composition
0.08	0.21	0.23	0.95
0.12	0.32	0.34	1.38
0.26	0.68	0.77	3.04
0.37	0.97	1.08	4.32
0.60	1.57	1.75	9.25

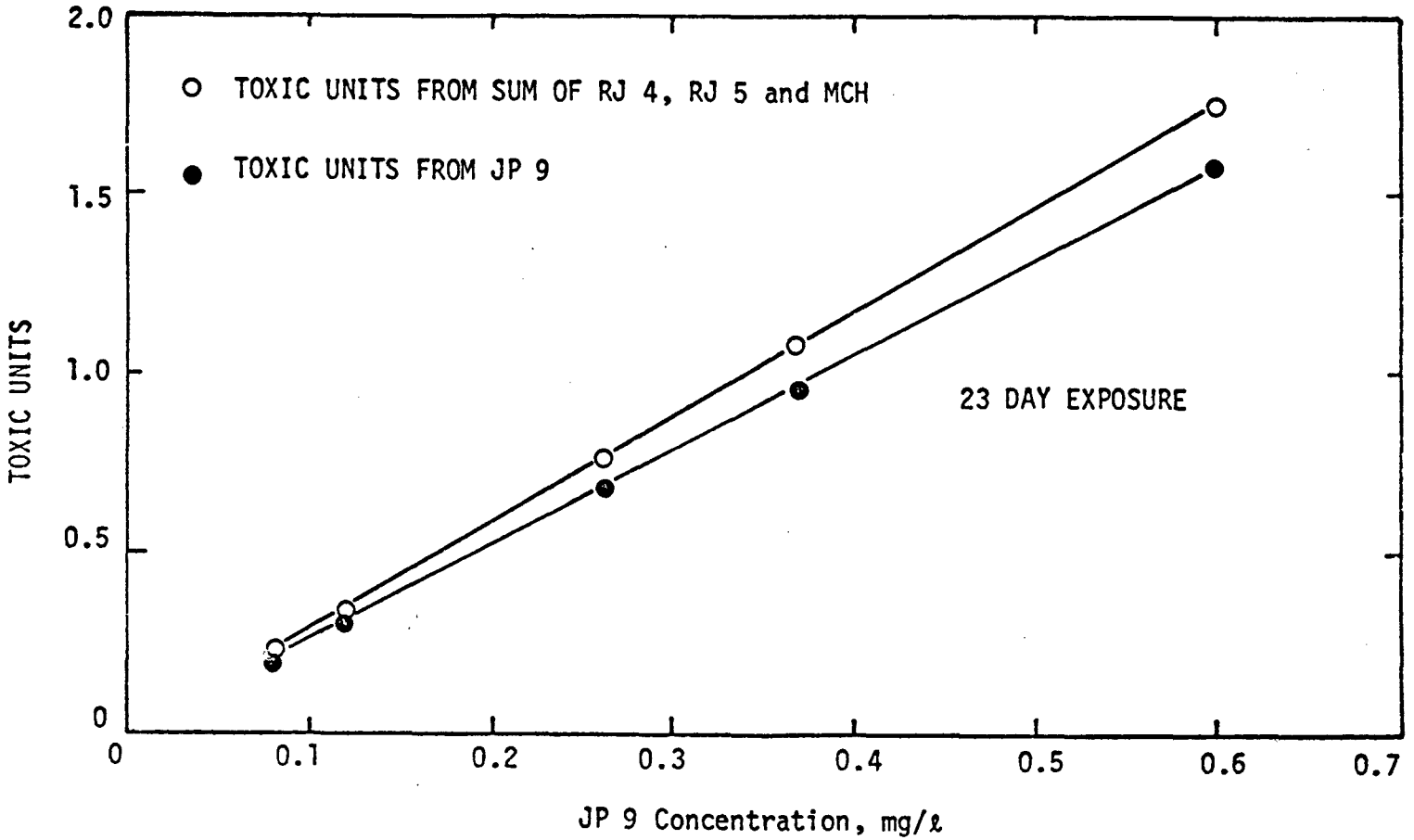


FIGURE 18. COMPARISON OF TOXICITY OF JP 9 AND COMPUTED TOXICITY OF ITS COMPONENTS. RAINBOW TROUT IN WATER SOLUBLE FUEL COMPONENTS, CONTINUOUS FLOW ASSAY

Fuel Accumulation—Surviving rainbow trout from the 97-day continuous bioassay of the soluble components of RJ4 and RJ5 were analyzed for their fuel content. The inclusion of a 2-5 sec pentane rinse prior to storage and extraction of the fish showed that only on the order of 0.1-0.2% of the total fuel (RJ4 or RJ5) found in the fish flesh could be accounted for on the surface of the fish. The rainbow trout were of a sufficient size to permit separation of muscle tissue and thus allow a comparison between whole body and muscle tissue burdens. The data in Figure 14 show that the total body burdens of RJ4 and RJ5 are related linearly to the aqueous RJ4 and RJ5 concentrations and that these fuels are concentrated in rainbow trout flesh to a much higher degree than is MCH in flagfish flesh. If one assumes that the single data point for MCH accumulation in rainbow trout (Figure 15) is valid, then the degree of concentration of RJ4, RJ5 and MCH in rainbow trout (assuming a wet fish density of 1.0) is respectively 9,800 times for RJ4, 3,900 times for RJ5 and 150 times for MCH. The RJ4 and RJ5 results indicate that the muscle tissue of rainbow trout contains approximately half the fuel concentration that the total body contains (Table 16). Since the aqueous RJ4 and RJ5 concentrations from which the fish for fuel analysis were taken covered a range of concentrations up to the LC 50 values, it is possible to make tentative statements concerning the fuel body burden that will cause 50% mortality. For RJ4 this concentration is on the order of 0.43 mg/g wet fish; for RJ5 this value is approximately 0.27 mg/g (Figure 19).

Table 16

ACCUMULATION OF RJ4 and RJ5 IN MUSCLE AND WHOLE BODY OF RAINBOW TROUT

Fuel	Mean Water Soluble Concentration mg/ℓ	Toxic Units	Fuel Concentration	
			Muscle mg Fuel/g Wet Fish	Whole Body mg Fuel/g Wet Fish
RJ 4	0.045	1	0.278	0.443
	0.025	0.56	0.133	0.217
	0.001	0.022	0.001	0.001
	0.08	1.1	0.186	0.311
	0.05	0.69	0.080	0.132
RJ 5	0.04	0.56	0.035	0.062
	0.003	0.04	0.003	0.003

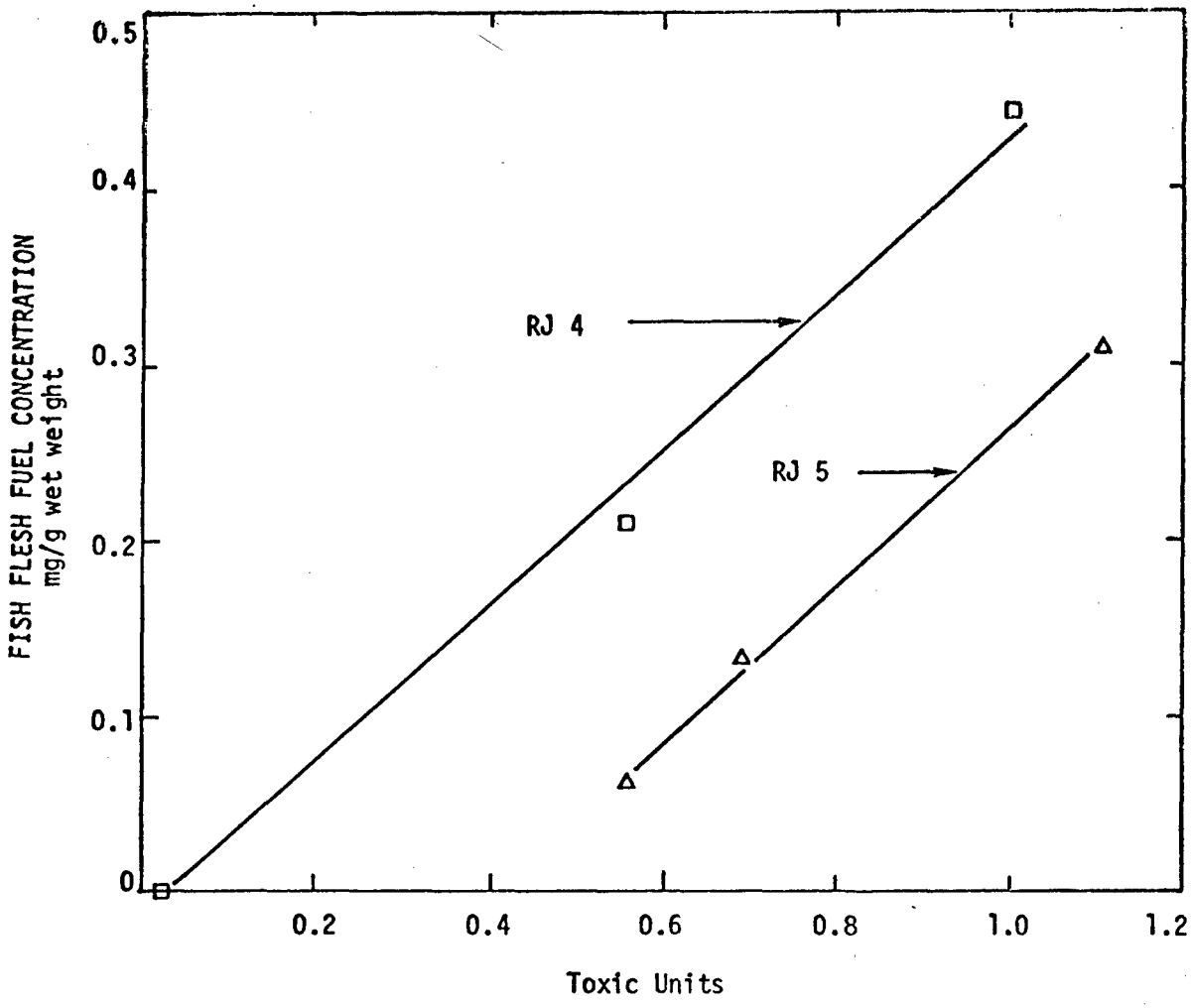


FIGURE 19. RELATIONSHIP OF FUEL BODY BURDEN TO TOXICITY OF WATER SOLUBLE RJ 4 AND RJ 5 TO RAINBOW TROUT

Voiding of Accumulated Fuel—The voiding of fuel components from rainbow trout was studied for MCH, RJ4 and RJ5. Surviving fish from the 97-day continuous bioassay of water-soluble components of these fuels were placed in fuel-free water (the control exposure tanks) and after known time intervals were sacrificed then rinsed and extracted with pentane. The total body burdens of fish from various fuel concentrations were normalized by using concentration factors (mg fuel/kg wet fish divided by mg fuel/ℓ exposure solution from which the fish was derived). These values, plotted in Figure 20, show that there was no evidence of any significant voiding of either RJ4 after 8 days or RJ5 after 6 days. However, the MCH level in the rainbow trout had dropped by a factor of almost 8 in a 12-hr period.

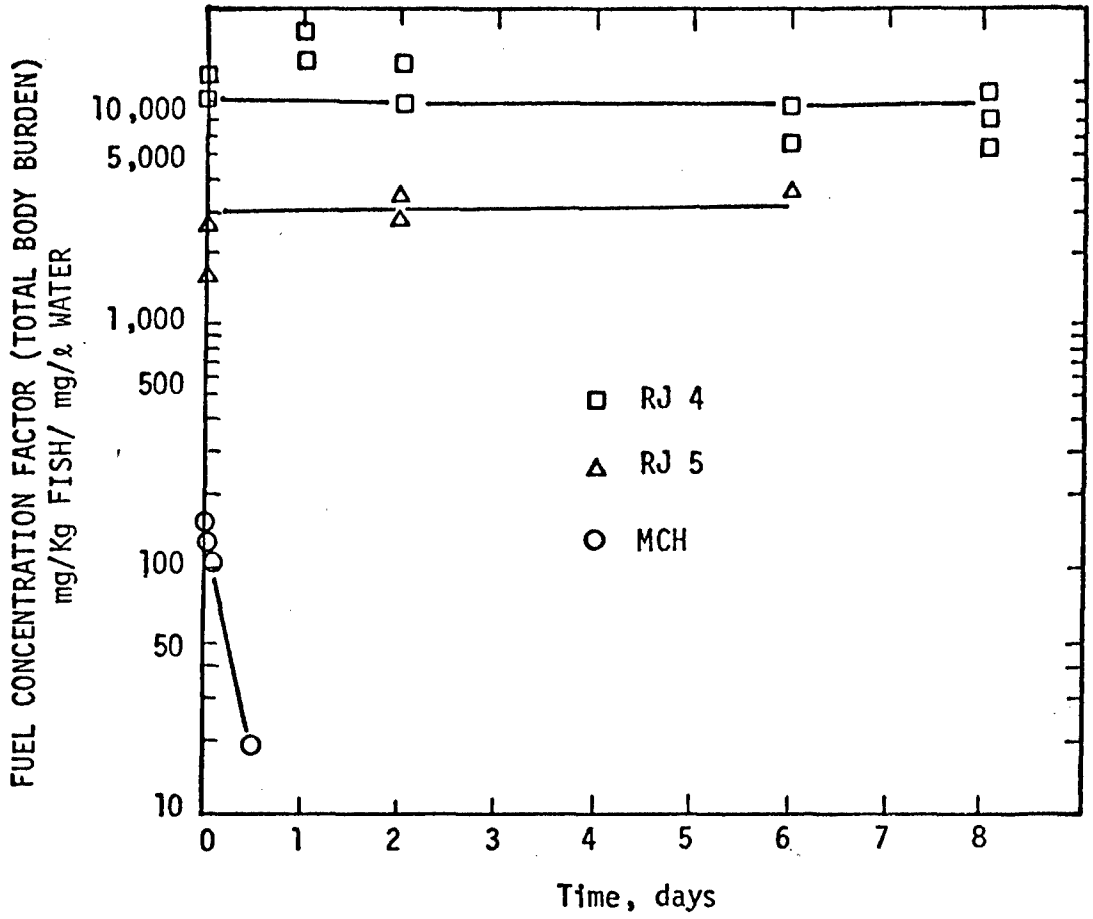


FIGURE 20. VOIDING OF FUEL COMPONENTS FROM RAINBOW TROUT

VI. STATIC AUFWUCHS BIOASSAY

Procedure

Growth units developed in the Mokelumne River were transported to the laboratory and subjected to the metabolic response test described in the Materials and Methods section. The aufwuchs were exposed to various dilutions of emulsified fuels and water saturated with fuels. Exposure of aufwuchs to pools of pure fuel was not investigated because (a) direct aufwuchs-fuel contact could not be avoided during the test procedure and (b) the short duration of the test combined with the relative insolubility of the fuels would not allow for a measurable response. Following a preliminary statistical evaluation of the response of control aufwuchs, experiments were conducted on emulsions of RJ4, RJ5 and JP4 and on water saturated with RJ4, RJ5, MCH and JP4. Planned experiments on other fuels could not be carried out because of seasonal constraints on development of aufwuchs. These studies were abandoned when aufwuchs growths were observed visually to be sparse and non-uniform. This situation is encountered most often in the winter when the cold and heavy ground fogs interfere with aufwuchs development and early spring when rapid river currents disturb the position of suspended racks.

Results

Statistical Evaluation of Aufwuchs Response—Thirty growth units from one rack were divided into three groups of 10 replicates. Chlorophyll *a* content and dry and organic weights were determined on the first group; the second group was used to assess the replication of the 2-hr light response; and the third to examine the variability of the 4-hr dark response.

The average sample standard deviation, s, the standard error of the mean, $SE_{\bar{x}}$, and the coefficient of variation, $C_V(\%)$, were computed for each group of measurements and for PI_{wt} and PI_{chl} (Table 17). Coefficients of variation of chlorphyll and dry and organic weights were in the range of 4-8%; the light response reproducibility was poor (C_V = 55.3%) and this measurement caused the PI_{wt} and PI_{chl} values to have poor reproducibility (C_V = 37% and 40.6%, respectively). Since the dark response measurement was significantly more reproducible (C_V = 16.7%), it is suspected that variable exposure of individual growth units in their respective BOD bottles may be the cause of the variability. It was noted that the BOD bottle stopper shielded aufwuchs from the overhead light source to a degree that varied with the position of the aufwuchs unit in the bottle.

This variability of response must necessarily introduce a significant degree of caution into the interpretation of the aufwuchs data and also indicate that a considerable amount of work needs to be done to improve the reproducibility of the aufwuchs technique. Notwithstanding, it is possible to discern significant trends in the response data for fuels presented below.

TABLE 17

STATISTICAL EVALUATION OF AUFWUCHS DETERMINATIONS IN THE ABSENCE OF FUELS

Statistical Measurements	Standing Crop					Photosynthesis and Respiration				
	Weights			Chlorophyll		mg O_2/Auf — hr			$PI_{wt.}$	PI_{Chl}
	Dry mg/Auf.	Organic mg/Auf.	Organic %	mg/Auf.	mg/g Org. wt.	Light (Net Photo.)	Dark (Resp.)	Gross Photo.		
	381	42	11.0	0.460	11.0	0.410	0.106	0.516	12.3	1.12
	337	40	11.9	0.462	11.6	0.070	0.100	0.170	4.3	0.37
	380	44	11.6	0.465	10.6	0.249	0.076	0.325	7.4	0.70
	358	39	10.9	0.508	13.0	0.198	0.083	0.281	7.2	0.55
	362	41	11.3	0.454	11.1	0.312	0.116	0.428	10.4	0.94
	342	39	11.4	0.508	13.0	0.332	0.083	0.415	10.6	0.82
	325	38	11.7	0.446	11.7	0.084	0.163	0.187	4.9	0.42
	322	35	10.9	0.457	13.1	0.123	0.086	0.204	6.0	0.46
	347	37	10.9	0.425	11.5	0.337	0.069	0.446	12.1	1.05
	344	37	10.7	0.451	12.2	0.120	0.079	0.199	5.4	0.44
Ave.	350	39	11.2	0.464	11.9	0.228	0.090	0.318	8.1	0.69
$\pm$ s	$\pm$20	$\pm$3	$\pm$0.4	$\pm$0.026	$\pm$0.9	$\pm$0.126	$\pm$0.015	$\pm$0.126	$\pm$3.0	$\pm$0.28
$SE_{\bar{X}}$	6	1	0.1	0.008	0.29	0.040	0.005	0.040	0.96	0.09
C_v, %	5.7	7.7	3.6	5.5	7.7	55.3	16.7	39.6	37.0	40.6

Emulsified Fuels—The results of three studies on JP4 and one study each on RJ4 and RJ5 is reported here. The aufwuchs studies on MCH and JP9 emulsions did not produce reproducible results.

The PI_{wt} data for JP4 emulsions (Table 18) can be linearized, with a correlation coefficient of -0.6 (Figure 21), in the JP4 concentration range from 1 to 1000 µℓ/ℓ as follows:

$$y = -0.09 \ln x + 1.2$$

where

$$y = \frac{PI_{wt} \text{ sample}}{PI_{wt} \text{ control}}$$

x = JP4 emulsion concentration, µℓ/ℓ correlation coefficient

Data for 10,000 µℓ/ℓ JP4/ℓ are omitted because of the interference of this high level of fuel emulsion with dissolved oxygen determination by the azide modification of the Winkler method. A red, oily layer (possibly iodine dissolved in fuel) formed in the BOD bottle during the measurement of dissolved oxygen. At 1000 µℓ/ℓ of emulsified JP4, there was an approximate 43% reduction in aufwuchs photosynthetic index. The first toxic effects (as assessed by reduction in photosynthetic index) can be discerned between 10-50 µℓ/ℓ JP4 emulsion.

The limited results for RJ4 and RJ5 emulsions (Figure 21) indicate similar toxicity to aufwuchs metabolism as JP4. At the 1000 µℓ/ℓ level, RJ4 suppressed PI_{wt} by 35% and RJ5 caused at 25% depression of PI_{wt} over respective control aufwuchs.

Water Saturated with Fuel—Water saturated with fuels and fuel components was far less suppressive of photosynthetic index than emulsified fuels (Table 19). Undiluted fuel-saturated water produced a 90% PI_{wt} for MCH (the most soluble fuel component tested), 59% reduction for JP4, 22% reduction for RJ5 and 3% reduction for the poorly soluble RJ4. These data tend to indicate a distinct relationship between fuel solubility and toxic effect of water extracts of fuel.

Discussion

Aufwuchs are known to be useful indicators of toxicity in saline estuarine waters where nutrients are plentiful and lush growths occur. More developmental work is clearly needed on fresh water aufwuchs determinations before toxicity measurements can be considered to be highly reliable. As indicated by the results presented herein, a correlation on the order of 60% can be achieved by conducting a series of studies on one fuel.

Table 18

TOXICITY OF EMULSIFIED JP4 TO AUFWUCHS

Study Number	Fuel Concn. µℓ/ℓ	Mean Growth per Aufwuchs Unit		Photosynthetic Index, PI	
		Organic Weight mg	Chlorophyll *a* mg/g org. wt.	PI_{wt} mg O_2/g Org. wt.-hr.	PI_{chl} mg O_2/mg chl *a*-hr.
1	0			49.0	2.5
	1			50.0	2.5
	10	21	19.7	49.0	2.5
	50			41.9	2.1
	100			41.9	2.1
2	0			12.2	0.68
	10			10.2	0.57
	100	36	18.0	6.89	0.38
	1,000			2.17	0.12
	10,000			-1.11	-0.06
3	0			7.9	0.57
	50			7.5	0.55
	200	±9.9	13.7	8.3	0.61
	400	0.8		5.4	0.40
	600			5.6	0.41
	800			5.6	0.41

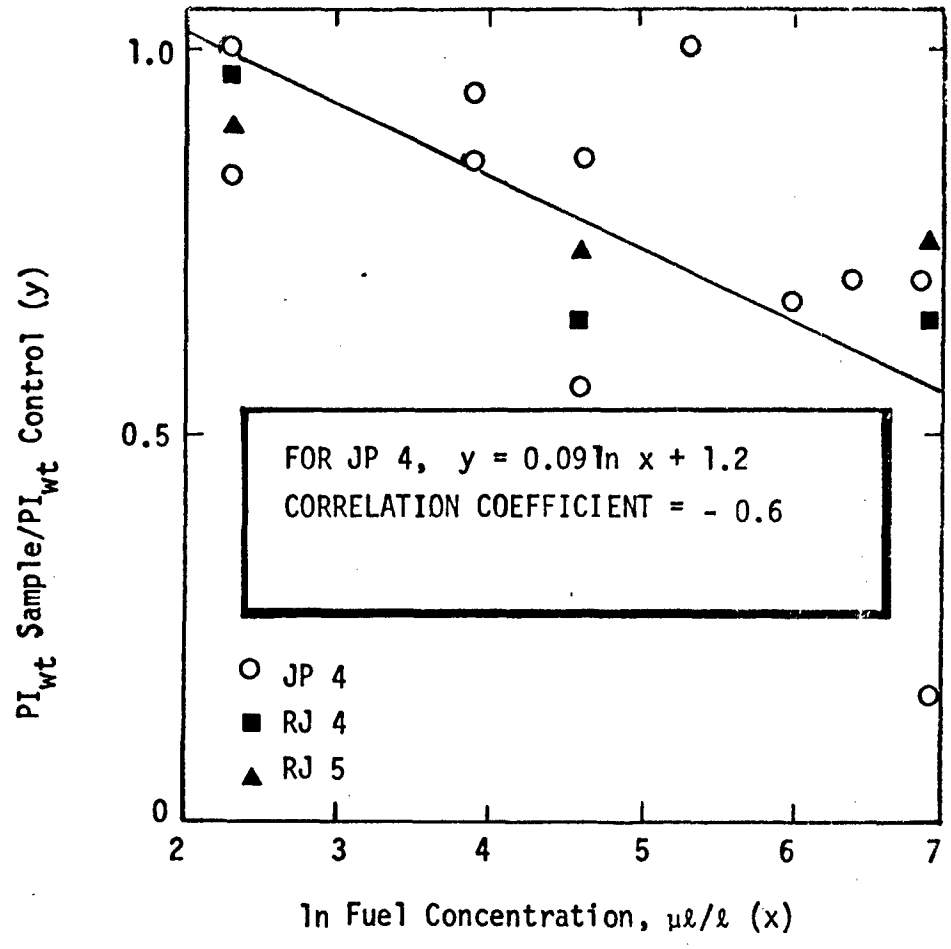


FIGURE 21. TOXICITY OF EMULSIFIED JP 4, RJ 4 AND RJ 5 TO AUFWUCHS

Table 19

TOXICITY OF WATER SATURATED
WITH FUEL TO AUFWUCHS

Water-Saturated Fuel	Mean Growth per Aufwuchs Unit		Photosynthetic Index, PI		
	Organic Weight mg	Chlorophyll a mg/g org. wt.	PI_{wt}	PI_{chl}	$\frac{PI_{wt}\ sample}{PI_{wt}\ control}$
Control	39±4	11.2	12.3	1.10	--
JP4			5.1	0.45	0.41
RJ4			11.9	1.07	0.97
RJ5			9.6	0.86	0.78
MCH			1.2	0.11	0.10

One change in technique that should produce significant improvement in methodology involves the use of a dissolved oxygen electrode rather than the Winkler method for oxygen determination. An oxygen electrode would enable the aufwuchs unit to be saved after the metabolic response test for direct measurement of mass or chlorophyll content rather than reliance on replication for obtaining these measurements. Obviously, this procedure would eliminate the error involved in non-uniformity of growth between individual aufwuchs units. The racks of units seldom contain uniform growths even in the summer months, and, as noted previously, the growths are very sparse in the winter and non-uniform in the spring.

The problem of light shielding by the BOD bottle stopper might be eliminated by laying the bottles on their side during the photosynthetic response test. Light intensity variation in the chamber is not a problem. Measurements have shown that the intensity varies only ±75 foot-candles from the mean of 1700 foot-candles. Based on the work of Ryther (1956), this variation would be expected to have a negligible effect on photosynthesis at this intensity level oxygen production.

Despite the problems in methodology, the results do offer indications of environmental impact with serious implications. The suppression of photosynthetic index by the water-soluble fractions of the fuels is of particular importance. This is most noticeable with the more soluble JP4 and MCH which appear to severely limit metabolic activity and could therefore affect the food chain of natural waters.

As noted in other discussions, the liklihood of emulsified fuels causing widespread problems in the aquatic environment is unlikely. However, the results indicate that dispersed fuel globules are toxic in the 100 to 1000 µℓ/ℓ range and could therefore retard periphyton growth in limited areas.

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